REGULATORY T CELLS IN RHEUMATOID ARTHRITIS: CLINICAL RELEVANCE AND MECHANISMS IN DISEASE

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ABSTRACT

The current work aimed to look at both phenotypic and functional differences in regulatory T cells (Tregs) from active rheumatoid arthritis (RA) patients compared to healthy controls and RA patients receiving infliximab therapy. There was an increase in the number $Foxp3^+$ Tregs in infliximab patients compared to healthy controls or active RA patients. Etanercept patients had similar Foxp3 levels to healthy controls. Active RA Tregs expressed significantly lower levels of CCR5, whilst CD40L was significantly up regulated. Analysis of CTLA-4 in active RA Tregs revealed differential results: CTLA-4 was up regulated on $CD4^+CD25^{hi}$ cells, but significantly down regulated on $CD4^+Foxp3^+$ Tregs. Furthermore, CTLA-4 remained low in infliximab-treated RA patients. Addition of recombinant TNF-α led to healthy Tregs phenotypically resembling active RA Tregs with respect to the markers described above.

Interestingly, Tregs from infliximab-treated patients were predominantly $CD62L^-$, in contrast to Tregs from active RA patients and healthy controls. $CD62L^-$ Tregs from infliximab patients were found to be more potent suppressors of T effector proliferation and cytokine production, than their $CD62L^+$ counterparts. Additionally, blockade of TGF-β and IL-10 abrogated their suppressive capacity, whereas healthy Tregs suppressed independently of these cytokines. Active RA Tregs and $CD62L^+$, but not $CD62L^-$ Tregs from infliximab patients produced comparable levels of IL-17.

In vitro addition of infliximab to RA T effectors induced functionally suppressive $Foxp3^+$ T cells, an effect not observed in healthy T effectors. Moreover, blockade of TGF-β in these *in vitro* cultures inhibited infliximab-induced $Foxp3^+$. Finally, analysis of the TGF-β signalling pathway in RA T effectors demonstrated that these cells expressed significantly lower levels of TGF-β receptor II, and decreased Smad2 phosphorylation, but not total protein.

Taken together, the current work demonstrates that active RA Tregs are phenotypically abnormal, which may partly explain their defective function. Moreover, infliximab therapy does not appear to simply restore the defect in the existing conventional Treg ($CD62L^+$) population, but rather induces a distinct population, which differs both phenotypically and functionally, suggesting that altering the pro-inflammatory environment is an important mechanism to restore tolerance.

DECLARATION:

I certify that this thesis does not incorporate any material previously submitted for a degree or diploma in any university, and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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INTRODUCTION

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1.1.1 POSITIVE AND NEGATIVE THYMOCYTE SELECTION

1.1.1.1 POSITIVE SELECTION

Following development to the single-positive (SP) stage, thymocytes undergo extensive selection pressures to ensure that mature T cells that are exported from the thymus into the periphery are self-MHC restricted. This ensures that they are self-tolerant, thereby limiting autoimmune reactions. Such processes are called positive and negative selection. Following these processes, developing thymocytes acquire a mature αβ TCR, during which TCRα gene rearrangement occurs.

In contrast to the TCRβ chain, the TCRα chain does not undergo allelic exclusion. Consequently, productive TCRα rearrangement may occur on both chromosomes, with the T cell potentially expressing two TCRα chains. However, since only one TCR is required during positive selection, the second TCR may not have affinity with self-MHC, and therefore would be non-functional. The TCRα chain comprises around 80 variable segments, each containing exons encoding a V region. The V region of the α chain only consists of V and J segments and rearrangement of genes is similar to rearrangement of TCRβ genes. Double-negative (DN) precursors that only express a TCRβ chain initiate TCRα locus rearrangement, which immediately follows pre-TCR signalling.

Following a second wave of RAG gene expression TCRα chain rearrangement commences, which lasts 3-4 days. Once double-positive (DP) thymocytes express αβ TCRs that have randomly generated antigen specificities, their TCRs are directed to peptides bound to a groove on cell-surface major histocomapatability complexes (MHC). MHC molecules are highly polymorphic, which enable T cells to recognise and respond to a broad range of

foreign peptides when they are in the periphery.

With respect to positive selection, however, thymocytes are stimulated to survive when their TCRs bind with low avidity (weakly) to self peptide-self MHC complexes and TCR recognition is augmented when CD4 and CD8 co-receptors bind to non-polymorphic sites on MHC. In contrast, thymocytes whose TCRs recognise self MHC complexes with high avidity are eliminated by apoptosis, in a process termed negative selection. This ensures that tolerance is maintained centrally and T cells that go onto maturation are self-MHC restricted.

At the point of αβ TCR formation, DP thymocytes express low levels of the antigen receptor, and are programmed to die unless they receive a survival signal generated by TCR contact with specific self peptide-MHC complexes. Peptides are generated in the thymus through the breakdown and processing of intracellular proteins. This enables MHC molecules expressed on cortical thymic epithelial cells (cTECs) to express a wide range of self-peptides.

Although the thymus generates large numbers of DP thymocytes, the majority of these cells have negligible affinity for self-peptide/MHC complexes and undergo apoptosis, a process termed 'death by neglect'. Consequently, only a small proportion of thymocytes (1-5%) that have significant binding specificity for self-peptide/MHC complexes go onto being positively selected. Such DP thymocytes receive a protective, low-level/weak TCR signal that allows thymocytes to survive, up-regulate their TCRs and differentiate into SP thymocytes. If the TCR recognises self peptide-loaded class II MHC molecules, and CD4 on the T cell interacts with this complex, then survival signals are received that promote its maturation. At this point, the CD8 co-receptor is down regulated, and the T cell becomes a MHC class II-restricted $CD4^+$ T cell. Similarly, if the TCR recognises self-peptide MHC class I complexes and CD8 interacts with the complex, then the T cell becomes MHC class I-restricted $CD8^+$ T cell.

von Boehmer proposed the instructive model for SP selection. In this model, MHC class I-specific TCR and CD8 co-receptor are co-engaged by the same MHC class I molecule, which subsequently generates signals that 'instruct' development of CD8 SP T cells and are distinct from MHC class II-engaged receptor (1). In another model, called the stochastic/selective model, lineage commitment occurs randomly by down regulation of CD4 or CD8 co-receptor surface expression, and the T cell expresses a TCR-co-receptor combination that "fits" the available MHC class I or II is selected for survival (1).

During positive selection, thymocytes transiently express the activation marker CD69, which discriminates between MHC-dependent and –independent stages of thymocyte positive selection (2). Most peptides that induce positive selection are encountered in the periphery, as well as the thymus. Thus, immature thymocytes undergoing positive selection will at some point re-encounter the same peptides at peripheral sites, such as the spleen and lymph nodes. Continuous encounters with these peptides have specific implications when the T cell enters the periphery. For example, when naïve T cells enter the periphery, they remain in an inactive/restive state until they encounter a foreign antigen. Therefore, continuous contact with self-peptides that induced positive selection in the thymus, ensure that T cells are ignored in the periphery and are allowed to mature in a post-thymic environment. This means that T cells are able to 'see' self-peptides in the thymus, but not in the periphery. The reason for this is that T cell maturation is associated with TCR desensitization, which prevents the T cell from recognising the original peptide that induced positive selection, whilst maintaining reactivity for foreign peptides. Positive selection, therefore, is required to generate mature T cells that have considerable specificity for self-peptides, but not react to self to cause autoimmune reactions.

1.1.1.2 NEGATIVE SELECTION

The elimination of T cells whose TCRs bind to self peptide/self MHC complexes with high avidity, is a process of central tolerance, called negative selection. Negative selection involves the apoptotic elimination of autoreactive T cells - i.e. T cells whose TCRs bind with a strong affinity to ubiquitous self-peptide/MHC complexes - and requirements for this process differ from positive selection, both in terms of cell types involved and the region of the thymus where the process takes place. Escape of such cells into the periphery would result in attack against self and induce autoimmune disease.

In contrast to positive selection, negative selection takes place within the thymic medulla, which is readily accessible to soluble antigens entering the thymus from the bloodstream. In addition, the medulla is densely packed with medullary thymic epithelial cells (mTECS) and antigen presenting cells (APCs), as well as bone marrow-derived DCs.

In vitro thymic organ cultures have shown that splenic dendritic cells are extremely effective at inducing tolerance in thymocytes. Moreover, comparison of tolerance induction by different cell subsets (such as splenocytes and thymocytes) revealed that splenic DCs are the most efficient, with just one DC inducing tolerance per 200 thymocytes *in vitro* (3). Several studies confirmed the above finding that although mTECs are able to induce negative selection in the absence of MHC class II, bone marrow derived DCs are much more efficient at inducing negative selection than mTECs (4, 5). Although medullary thymocytes are SP T cells, they are not fully mature, and the majority of these semi-mature T cells resemble DP thymocytes in that they express the heat-stable antigen (HSA) (6).

Co-stimulatory molecules play an important role in negative selection. *In vitro* cross-linking of purified DP thymocytes with plate-bound anti-TCR mAb alone did not induce apoptosis. However, when DP thymocytes are cross-linked with anti-TCR in conjunction with anti-CD28, apoptosis was rapidly induced (7, 6). However, it seems that there is no single co-stimulatory molecule responsible for inducing negative selection. For example, $CD28^{-/-}$ mice display normal negative selection processes (8), suggesting that other co-stimulatory molecules take over in the absence of CD28, such as CD5 and CD43 (9).

CD40L (gp39) is also implicated in negative selection, and it has been demonstrated that binding of CD40L to its receptor, CD40, is required to up-regulate B7-2 expression on thymic medullary cells, which coincides with induction of negative selection. This therefore suggests that CD40L aides negative selection by regulating expression of co-stimulatory molecules on thymic medullary epithelium (10).

The medulla is the site for the final stages of lymphocyte development within the thymus, which is characterised by the maturation of SP thymocytes. This final maturation process is accompanied by further deletion of self-reactive thymocytes that are by-passed in the cortex. This additional deletion process is important in establishing central tolerance to tissue-specific antigens. Such antigens include myelin basic protein, insulin and thyroglobulin, which are associated with the organ specific autoimmune diseases multiple sclerosis, type 1 diabetes and thyroiditis, respectively.

The expression of tissue-specific antigens by mTECs is partially regulated by the transcription factor autoimmune regulator (AIRE). Deficiency in AIRE causes failure in establishing central tolerance, and humans with mutations in the *AIRE* gene suffer from a multi-organ disorder called autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APCED).

AIRE promotes the expression of tissue-specific antigens by regulating their transcription, and therefore alters gene expression by mTECs (11). Not all tissue-specific antigens are regulated by AIRE, since mTECs from AIRE-deficient mice still express C-reactive protein (CRP) and GAD67, which are liver- and pancreas-specific proteins, respectively (11).

Although negative selection is an efficient process at maintaining central tolerance, some self-reactive T cells are still able to escape deletion and enter the periphery, since not all self-antigens are expressed in the thymus. Therefore, peripheral tolerance mechanisms are present, which make sure T cells that encounter their first self-antigen outside the thymus, become tolerant.

1.1.2 NATURAL REGULATORY T CELLS AS A DISTINCT T CELL POPULATION - EARLY EXPERIMENTAL EVIDENCE

Early experiments on mice demonstrated the importance of the thymus in controlling autoimmunity. In these experiments, thymectomy on 3 day old female mice resulted in infertility and an organ-specific autoimmune reaction to the reproductive organs (12). Additionally, if mice are thymectomised (Tx) on day 1 or day 3 (post natal), reproductive organs remain healthy. Moreover, grafting of thymuses from day 1 or day 7 mice into day 3 Tx mice prevents the autoimmune destruction of the reproductive organs.

The results of these early experiments therefore suggested that a regulatory population of T cells existed that originated within the thymus. Subsequent experiments clearly demonstrated that a specific population of T cells are present within the thymus, which are capable of preventing multi-organ specific autoimmune disease. T cells do not migrate into the periphery (spleen), until after day 3 postnatal, and thymectomy of nude female mice at this time point leads to the development of oophoritis (inflammation of the ovaries).

Adoptive transfer of splenocytes from Tx mice into normal newborn mice results in the development of oophoritis in these mice, and further investigation, via anti-sera experiments, have shown that Lyt-1 (CD5 antigen), but not Lyt-2/3 (CD8 antigen) were responsible for disease (13). These experiments therefore suggested that neonatal (day 3) thymectomy of mice inhibits the development of T cells that are capable of inhibiting autoimmune disease.

A second set of experiments, had shown that if splenocytes from normal adult mice are transferred into neonatal Tx mice, then autoimmune disease is prevented, and the cell type that confers protection are $CD5^{+}CD8^{-}$ (14). These series of experiments sequentially identified a population of T cells with suppressor activity. However, one problem of using CD5 antigen as a marker for a putative regulatory/suppressor T cell population is that it is expressed on all T cells.

Subsequent experiments described a subset of $CD4^{+}CD45RB^{low}$ T cells that are able to exert an inhibitory action on the $CD4^{+}CD45^{high}$ fraction. A wasting disease, coupled with multi-organ pathology is observed in athymic rats that received small numbers of $CD4^{+}CD45RB^{high}$ cells, but disease progression is prevented when athymic rats were injected with $CD4^{+}CD45RB^{low}$ T cells (15). These findings were later confirmed in severe combined immunodeficient (SCID) mice (16).

The observation that 10% of $CD4^{+}$ T cells are functionally suppressive when they co-express the IL-2 receptor α chain (CD25) demonstrated that a definitive population of regulatory T cells (Tregs) exist naturally, originating in the thymus. These $CD4^{+}CD25^{+}$ T cells correlate with a $CD5^{+}CD45RB^{low}$ phenotype, and thereby confirmed the original findings described above. Transfer of spleen suspensions from normal mice depleted of the $CD4^{+}CD25^{+}$ subset, into athymic mice coincides with multi organ-specific autoimmune disease, including gastritis, oophoritis, and thryroiditis. By contrast, transfer of cell suspensions enriched with $CD4^{+}CD25^{+}$ prevents autoimmune pathology (17).

1.1.3 CO-STIMULATION AND NATURAL REGULATORY T CELLS: ASSOCIATION BETWEEN PHENOTYPE AND FUNCTION

The process of activation and differentiation of naïve T cells, including Tregs, is called priming and relies on a combination of stable interactions with the dendritic cell via the MHC-peptide complex. These interactions occur at the immunological synapse (IS), a nanometre gap that forms between a T cell and a DC and is enriched with specific receptors. Although the initial activating (or inhibitory) interaction at the IS is rapid (within seconds), the required duration of signalling is much longer, and can last several hours.

Priming of naïve T cells is a two-step process commonly referred to as the two-signal hypothesis: signal one is generated by initial TCR/CD3 interactions with the MHC-peptide complex, which activates the T cell. Signal two is generated by co-stimulatory molecules, such as CD28 and CD40L (on T cell) binding to its receptors on DC (B7 molecules and CD40, respectively), which provides a survival signal for the T cell. A third signal is also required to produce effector subsets of CD4 T cells, and is usually supplied by cytokines, which are involved in T cell differentiation.

Natural regulatory T cells (Tregs) – i.e. those that are derived from the thymus - make up 1-10% of the total $CD4^+$ T cell population, and by definition, are anergic in that they do not proliferate in response to TCR activation. This state of anergy by Tregs has been shown to only occur *in vitro*, and if this anergic state is broken, for example by the addition of high concentrations of IL-2, the suppressive capacity of the Treg is lost. However, while Tregs are anergic *in vitro,* they are able to proliferate following TCR stimulation in vivo (18).

Natural Tregs function via a cell-contact dependent mechanism, which include interactions between the co-stimulatory molecules CD28 and cytotoxic lymphocyte antigen-4 (CTLA-4; CD152) with their ligands B7-1 and B7-2 (CD80, CD86, respectively) and CD40L (CD154; gp39) and CD40 on APC and the tumour necrosis factor (TNF) superfamily member, glucocorticoid-

induced TNF receptor, GITR. Evidence for this comes from *in vitro* experiments: in the presence of a semi-permeable membrane, thereby separating and preventing contact between the Treg and T effector ($CD4^+CD25^-$) populations, Treg-mediated suppression is abrogated (19).

Similarly, experiments using supernatants collected from cultured Tregs have shown that soluble factors are not responsible for Treg- mediated suppression, since supernatants from these cells are unable to suppress effector T cells (20). In order to induce their suppressor function, Tregs need to be stimulated through their TCR, either in an antigen-dependent manner, or through polyclonal stimulation (19), enabling them to suppress in a broad, antigen non-specific manner.

Like their murine counterparts, human thymic Tregs are also anergic *in vitro* and fail to produce cytokines such as IL-2, IL-4, IL-10 or IFN-γ. The anergic phenotype is lost in the presence of exogenous IL-2 and IL-4 (21). Suppression occurs in an antigen non-specific cell-contact dependent manner, since neutralisation antibodies to both IL-10 and TGF-β has been shown not to affect Treg suppression, whereas the presence of a semi-permeable membrane does (21, 22).

The development of Tregs within the thymus is dependent on an intact IL-2-IL-2R pathway: Studies looking at IL-2Rβ$^{-/-}$ mice showed that these mice had no $CD4^+CD25^+$ T regs and experienced autoimmune lethality. However, when an intact IL-2Rβ transgene is introduced, the defect was reversed (23). This suggests that IL-2 is required, at least, for the development of Tregs, and the role of the cytokine extends to the periphery, where it is needed for the maintenance of Treg homeostasis. Phenotypically, Tregs resemble activated T cells, constitutively expressing high levels of a number of surface markers. Such markers include the IL-2Rα chain, CD25, as well as expression of surface markers CD62L (L-selectin), CD103 (αEβ7) and CD45RO.

1.1.3.1 CD28-MEDIATED CO-STIMULATION

CD28 is a 44-kDa integral membrane glycoprotein, which consists of an extracellular Ig-like domain and a short (41 amino acids in length) cytoplasmic tail that lacks intrinsic catalytic activity. Therefore, the cytoplasmic tail is associated with phosphotyrosine kinases (PTKs) such as Lck and Fyn to phosphorylate CD28. The cytoplasmic tail also contains a highly conserved Tyr-Met-Asn-Met (YMNM) motif. Phosphorylation of the tyrosine residue in this motif results in the recruitment of specific signalling molecules, including PI3K. CD28 is constitutively expressed in 90% of human $CD4^+$ T cells and 50% of human $CD8^+$ T cells. The ligands for CD28 are the B7 molecules, CD80 (B7-1) and CD86 (B7-2), which are structurally homologous and expressed at high levels on mature DCs (and other professional APCs such as macrophages and B cells). Although CD80 is absent in non-activated DCs, CD86 is expressed at low levels.

CD28-mediated co-stimulation is important in a number of T cell responses, including T cell proliferation and IL-2 production, which is necessary for T cell survival. In addition to T cell survival and proliferation, CD28-mediated co-stimulation results in a variety of other T cell responses, including IL-4 production, which induces differentiation of $CD4^+$ T cells into T-helper type 2 (Th2) cells (24); formation of the B cell areas in secondary lymphoid organs i.e. the germinal centres (25). Use of CD28 deficient mice has shown that CD28-mediated co-stimulation is also required for B cell isotype switching, since these mice have low levels of both IgG1 and IgG2a (26).

Although CD28 signalling promotes T cell activation, it simultaneously promotes negative T cell regulation. Following CD28 co-stimulation, CTLA-4 (a member of the CD28 family; see next sub-section) is rapidly up regulated, and antagonises CD28-mediated signals. In addition to CTLA-4, CD28 signals up regulated another CD28 family member, inducible co-stimulator (ICOS).

CD28 AND NATURAL REGULATORY T CELL HOMEOSTASIS

CD28 and CTLA-4 are both able to bind to the B7 molecules, but have opposing effects upon binding. CD28 is constitutively expressed on T cells, and binding to the B7 molecules results in activation of the immune system. CD80/CD86-CD28 interaction is required for regulation of Treg development. *In vivo* studies looking at $CD28^{-/-}$ and $CD80/CD86^{-/-}$ non-obese diabetic mice (NOD; a disease model for type 1 diabetes), showed that these mice had significantly lower numbers of Tregs compared to wild type. However, Treg function was not impaired (27), demonstrating the requirement for CD28 co-stimulation for the thymic development of Treg. Peripherally, CD28 co-stimulation is required for Treg homeostasis, since *in vivo* proliferation and survival of Treg is inhibited following blockade of CD80/CD86 (28). These results suggest that CD28, in addition to contributing to central tolerance during thymic development of Tregs, plays an important role in the maintenance of peripheral homeostasis of these suppressor cells.

1.1.3.2 CO-INHIBITORY SIGNALS MEDIATED BY CTLA-4

CTLA-4 (CD152) belongs to the CD28 family of receptors. Unlike CD28, which is uniformly distributed around the plasma membrane, CTLA-4 is present within intracellular vesicles at the tail end of the cell prior to activation. Upon activation (and therefore increasing TCR signal strength), *de novo* transcription of CTLA-4 occurs, and cytoskeletal reorganisation promotes the reorientation of CTLA-4-containing vesicles to the leading edge of the T cell, bringing them into close proximity to APCs, thereby allowing CTLA-4 to cycle to the surface at the immunological synapse (29).

The intracellular storage of CTLA-4 means that trafficking to the plasma membrane is tightly regulated. Upon activation, CTLA-4 leaves the Golgi apparatus and is transported to the plasma membrane via the adaptor protein, AP-1. CTLA-4 binds to AP-1 via a specific YVKM motif situated within CTLA-4's cytoplasmic tail (30). Trafficking of CTLA-4 to the plasma

membrane is regulated by phospholipase D (PLD) and ADP-ribosylation factor (ARF)-1(31). The clathrin-associated adaptor protein, AP-2 (specifically the μ2 subunit), regulates surface expression of CTLA4 by transporting it to the lysosomes for degradation via endocytosis. Surface expression of CTLA-4 is also regulated by a tyrosine residue in the YVKM motif, phosphorylation of this residue either by signals generated by Lck or TCR signals via Zap-70, retain surface expression of CTLA-4, and prevents its subsequent endocytosis (32).

CTLA-4 is differentially expressed on the surface of T cells, compared to CD28. While CD28 is constitutively expressed on resting and activated T cells, CTLA-4 is only expressed on T cells, following their activation through CD28-CD80/CD86 co-stimulation. However, CTLA-4 is constitutively expressed on murine Tregs (33), and is also observed on the surface of human Tregs (34).

Original experiments suggested that the ligands for CD28 and CTLA-4, CD80 and CD86, bind with equal affinities to their receptors (35). However, recent studies have demonstrated that the CD28 and CTLA-4 bind to their ligands with differing affinities: CTLA-4 binds to both CD80 and CD86 with a 100-fold greater affinity than CD28; CTLA-4 binds preferentially to CD80 over CD86 and with higher affinity than CD28 binding to CD80. Moreover, compared to its binding affinity with CTLA-4, CD86 binds to CD28 3-times more effectively than CD80. This suggests a co-stimulatory bias, whereby under normal conditions, expression of CD80 would favour CTLA-4-mediated responses; in contrast, CD86 on APCs would favour CD28-mediated responses (36).

These findings are mirrored by recent findings which show that expression of CD86 preferentially recruits CD28 to the immunological synapse, whereas CD80 expression preferentially recruits CTLA-4 (37). This would favour optimal T cell activation, since CD86 is constitutively expressed on APCs, whereas CD80 is up regulated following activation, and therefore would serve to enhance CTLA-4-mediated inhibitory signals.

CTLA-4-MEDIATED SIGNALLING

There are several mechanisms by which CTLA-4 may function, which fall into two categories: cell intrinsic, where CTLA-4 exerts its effects directly on the T cell; or cell extrinsic, where CTLA-4 may exert its effects via other cells, including APCs and Tregs. Evidence for cell intrinsic effects of CTLA-4 come from original experiments demonstrating that cross-linking of CTLA-4 with CD3 and CD28 results in significant reduction in T cell proliferation that coincides with attenuated IL-2 production, an effect that is not seen with CD3 and CD28 co-stimulation alone (38). Other cell intrinsic effects by CTLA-4 include regulation of signalling molecules involved in TCR stimulation. For example, association of CTLA-4 with the TCRζ chain recruits SHP-2, a CTLA-4-associated phosphatase that dephosphorylates the ζ-chain, and consequently inhibits TCR signalling (39).

Extending on these findings, it has also been demonstrated that CTLA-4 regulates the distribution of phosphorylated TCRζ within lipid rafts: in the absence of CTLA-4, the TCRζ chain accumulates within rafts, thereby allowing normal T cell activation to occur; however, if CTLA-4 is associated with the TCR at the point of T cell activation, the TCRζ chain is dissociated from lipid rafts (40). These studies indicate that cell intrinsic effects of CTLA-4 function to inhibit T cell proliferation by promoting inhibitory/negative signals. However, the timing of CTLA-4 expression, i.e. following T cell activation, suggests that CTLA-4 is actually involved in cell extrinsic-mediated inhibition.

Evidence for this comes from *in vivo* experiments in which $RAG2^{-/-}$ mice that have received $CTLA\text{-}4^{-/-}$ splenocytes, develop severe and fatal lymphoproliferative disease; however, if $RAG2^{-/-}$ mice have received $CTLA\text{-}4^{-/-}$ splenocytes, together with wild-type splenocytes, then disease is averted and the mice survive, suggesting that CTLA-4 indirectly inhibits self-reactive T cells (41).

Other evidence that favours cell extrinsic effects of CTLA-4 is its regulation of the enzyme indoleamine-2,3-dioxygenase (IDO), which is expressed on APCs and breaks down tryptophan, a key amino acid that promotes T cell proliferation. Expression of IDO is induced by IFN-γ, which enhances transcriptional activity of IDO via STAT1 (42)

CTLA-4 AND NATURAL TREG FUNCTION

The importance of CTLA-4 in promoting tolerance is seen in CTLA-4-deficient mice. These mice have increased numbers of self-reactive T cells that cause severe lymphoproliferative disease together with lethal multi-organ tissue destruction (43, 44). Since CTLA-4-deficient mice undergo normal thymic development (45), this suggests that the pathology seen in these mice results from failure of peripheral tolerance of T cells.

In contrast to CD28, CTLA-4 is induced de novo, following T cell activation, and is an important negative regulator of T cell activation. However, CTLA-4 may not necessarily function just to provide a negative signal, but rather enhance the suppression by Tregs. Under non-activated conditions, CTLA-4 is constitutively expressed in intracellular compartments of Tregs, and is only expressed on the cell surface following activation. The strength of TCR signal has been shown to regulate the surface expression of CTLA-4: Strong TCR signals up-regulate CTLA-4 to the surface, where it can interact at the immunological synapse (29). This would fit in with Treg function, since they possess high-affinity, self-reactive TCRs.

The consensus of the essential role of CTLA-4 in Treg function is challenged in studies looking at CTLA-4$^{-/-}$ mice: if CTLA-4 is blocked in wild-type mice, then Treg-mediated suppression is abrogated; however, CTLA-4$^{-/-}$ mice are able to develop functionally suppressive Foxp3^{+} T cells, which coincides with increased levels of IL-10 and TGF-β (46). This suggests that CTLA-4 is necessary for Treg function if it is already present on the Treg, but if CTLA-4 is absent at the time of thymic development, other compensatory mechanisms take place that ensure Treg function is not affected.

Expression of the CD80/CD86 molecules is not restricted to APC. Constitutive expression of CD86 has been found to occur on the surface of resting T cells (47). The resultant T-T (as opposed to T cell-APC interactions) interaction has important implications in immune regulation. T effector-expressed B7 engagement to Treg is important in conveying negative signals - Treg function is not maintained in mice whose T effectors lack both CD80 and CD86. However, Treg function is maintained when CD80/CD86 expression on T effector is restored. Interestingly, Treg function is also maintained even when the B7 molecules lack the parts of both the transmembrane and cytoplasmic domains, which are important in binding to CTLA-4, again suggesting that compensatory mechanisms are induced (48).

1.1.3.3 GITR-MEDIATED CO-STIMULATION

Glucocorticoid-induced TNF- related protein (GITR, TNFSR18) is a member of the TNFR superfamily. GITR is expressed on most T lymphocytes, as well as NK cells and DCs, functioning as a co-stimulatory molecule. GITR is activated by its ligand, GITRL, which is expressed mainly on APCs, and is down regulated following toll-like receptor-mediated stimulation (49) . The levels of GITR expression depend on the activation status of the cell. The receptor is constitutively expressed, at low levels, on freshly isolated $CD4^+$ and $CD8^+$ T effectors, and following stimulation, via the TCR, GITR is up regulated (50, 51). Kinetic studies looking at GITR expression have shown that optimal expression occurs less than 24 hours following TCR stimulation, suggesting that GITR expression is rapidly up-regulated following TCR stimulation (51). However, suboptimal TCR stimulation greatly enhances the kinetics and level of GITR expression. Moreover, co-stimulation via CD28 augments GITR expression, whereas blockade of CD80 and CD86 inhibits GITR expression, suggesting that the two co-stimulatory molecules work in synergy (51).

Co-stimulation via GITR-GITRL interactions augments TCR-induced T cell proliferation. Additionally, GITR-mediated co-stimulation up-regulates cytokine production, including IL-10, IFN-γ, IL-2 and IL-4 (51, 52). The cytokine profile that is up regulated following GITR-mediated co-stimulation reflects the protective effect GITR interactions can have in certain immune reactions. For example, GITR-mediated co-stimulation augments immune responses to viral pathogens, via increased levels of $CD8^+$ T cell-derived IFN-γ (53, 54). GITR-mediated co-stimulation also enhances immunity to tumour pathogens, not only by augmenting $CD8^+$ T cell responses (55), but also by preventing the infiltration of $Foxp3^+$ Tregs into tumour sites (56). Although a protective role for GITR has been demonstrated, GITR-GITRL interactions have also been shown to exacerbate autoimmune reactions, including EAE (57). Additionally, GITR-GITRL interactions exacerbate both asthma and collagen-induced arthritis by up-regulating expression of Th1 and Th2 transcription factors, T-bet and GATA-3, resulting in increased levels of IFN-γ, TNF-α, IL-4 and IL-5 (58).

GITR-GITRL INTERACTIONS AND REGULATORY T CELL FUNCTION

GITR is constitutively expressed on Tregs; however, unlike T effector cells, Tregs express high levels of GITR on their surface (59, 50), and $GITR^{-/-}$ mice still develop Tregs, suggesting that GITR is not necessary for their development (52). Unlike CTLA-4, which promotes Treg function, GITR has been shown to reverse the suppressive capabilities of these cells. The addition of agonistic GITR-specific antibody (clone DTA-1) to *in vitro* cultures containing Tregs and T effectors abrogates Treg-mediated suppression (59, 50).

Although the consensus is that GITR counteracts Treg suppression, the way in which the co-stimulatory molecule is believed to achieve this differs. For example, one study suggests that GITR directly inhibits Treg activity: by using rat T effector cells (which are not responsive to anti-mouse GITR antibody), it was shown that addition of GITR Ab to co-cultures containing rat T effector cells and mouse Tregs resulted in increased T cell proliferation, suggesting that the agonistic GITR Ab inhibited Treg function (50). In another study, however,

engagement of GITR on T effector cells, but not Tregs, with GITRL, was shown to abrogate T effector suppression, suggesting that GITR-mediated co-stimulation enhances T effector resistance to Treg suppression, rather than directly affecting the suppressive capability of Tregs (49).

While some studies have shown that GITR-mediated co-stimulation does not affect the anergic state of Tregs (50), it has been also been demonstrated that GITR-mediated co-stimulation, in the presence of IL-2 leads to the expansion of Foxp3-expressing Tregs (59, 60). Moreover, following GITR stimulation, these expanded Tregs are functionally suppressive (60). Taken together, these results suggest that while the immediate effect of GITR-mediated co-stimulation is to inhibit Treg suppression, the long-term effects actually promote Treg suppression.

1.1.3.4 Co-stimulation via CD40-CD40L interactions

CD40L is a 39kDa integral membrane protein that is expressed on activated, but not resting T cells, as well as activated B cells, platelets, peripheral blood monocytes, and human vascular endothelial cells. The receptor for CD40L is the 48 kDa transmembrane glycoprotein, CD40, which shares sequence homology with the TNFR family. CD40 is expressed on B cells, and activated DCs, macrophages, epithelial cells and vascular endothelial cells. Ligation of CD40L to its receptor results in the induction of thymus-dependent (TD) humoral responses. In addition to humoral responses, CD40L-mediated co-stimulation is important in DC maturation and subsequent licensing to prime naïve T cells. As mentioned above, DC maturation relies on toll-like receptor (TLR)-mediated signalling. However, DC maturation also depends on signals derived from CD40L-CD40 interactions, therefore, synergy between the two signalling systems is important in triggering cell-mediated immune responses. For example, CD40-mediated co-stimulation alone is insufficient to induce IL-12 production from DCs, and requires additional signals mediated by co-engagement of TLRs (61).

CD40-CD40L INTERACTIONS AND NATURAL TREG DEVELOPMENT AND FUNCTION

CD40-mediated co-stimulation is also important in Treg development: CD40-CD40L interactions are important during thymic development, as demonstrated in $CD40^{-/-}$ mice who have significantly reduced numbers of Tregs, together with an increase susceptibility to autoimmunity (62). CD40-mediated co-stimulation has differing effects in the peripheral regulation of Tregs: blockade of CD40L results in prolonged graft survival in recipient mice. In addition, Tregs from $CD40L^{-/-}$ mice experience greater graft survival compared to wild-type mice (63). However, transfer of wild-type Tregs into $CD40^{-/-}$ mice results in significant reduction in development and survival of these cells, coupled with reduced IL-2 production. Moreover, Tregs from $CD40^{-/-}$ mice are still functionally suppressive (64). Taken together, these results suggest that CD40-CD40L interactions are required for thymic Treg development, but are dispensable for Treg function.

CD40-CD40L interactions have been shown to interfere with Treg suppression: blockade of CD40L results in prolonged graft survival in recipient mice. In addition, the Tregs from $CD40L^{-/-}$ mice have much higher graft survival compared to wild-type mice (63) An earlier study looking at the effect of blocking CD40-CD40L interaction in GvHD, showed that rather than inducing tolerance, the blockade of such associations prolong graft survival. This was suggested to be due to the fact that blockade of CD40L did not induce tolerance of $CD8^+$ T cells, but only the $CD4^+$ population (65). A more recent study looking at $CD8^+$ T cell-mediated GvHD showed that upon CD40L blockade, tolerance can be induced if Tregs are present in sufficient numbers (66). These studies therefore suggest that CD40-CD40L interactions have differing roles in Treg function and development, depending on the site of the interaction: CD40-CD40L appears to be important during thymic development, but abrogates Treg function once the cells are in peripheral sites.

1.1.4 NATURAL REGULATORY T CELLS IN HUMANS

Human Tregs are similar to murine Tregs both phenotypically and functionally. Additionally, they are similar to their murine counterparts in terms of their anergic phenotype (at least *in vitro*) to stimulation by TCR cross-linking in the absence of exogenous IL-2. However, there are significant differences between human and murine Tregs, in terms of CD25 expression. Whereas murine Tregs have a distinct population of $CD4^+$ Tregs that are $CD25^+$, human CD25 expression within the total $CD4^+$ T cell population differs greatly, mainly due to the fact that humans are constantly in contact with, or have been previously exposed to various microbial products.

Expression of CD25 on $CD4^+$ T cells varies: between 2-5% $CD4^+$ T cells express 'high' levels of the IL-2Rα chain, with another 30% expressing 'low' levels of CD25. Therefore, human Tregs cannot be distinguished simply as $CD4^+CD25^+$, as this may incorporate a significant population of activated T cells. Within the $CD25^+$ population, only $CD4^+$ T cells expressing high levels of CD25 ($CD25^{hi}$) are functionally suppressive (67).

Analysis of different surface markers, including CD45RO, the lymph node homing molecule CD62L, and the IL-2Rβ chain CD122, has shown that a more homogenous population lies within the $CD4^+CD25^{hi}$ population, with 95% expressing the above markers. In contrast, analysis of the $CD4^+CD25^{lo}$ population has shown a more heterogeneous phenotype (67). Other markers expressed by human Treg are similar to those expressed by their murine counterparts, and include CTLA-4 and GITR.

Co-stimulatory molecule	Expression on Treg surface	Required for Treg thymic development?	Required for Treg homeostasis?	Effect on Treg function
CD28	Constitutive	Yes	Yes	No effect
CTLA-4	Constitutive/ high	No	Yes	Enhances suppression
GITR	Constitutive/ high	No	No	Inhibits initial Treg suppression; expand suppressive Tregs
CD40L	Low in periphery	Yes	No	Abrogates peripheral Treg function

TABLE 1.1.3 EXPRESSION OF CO-STIMULATORY MOLECULES ON NATURAL REGULATORY T CELLS

The expression of specific co-stimulatory molecules on the surface of regulatory T cells are required for their thymic development, as well as peripheral homeostasis and function. The effect on regulatory T cell function depends on the co-stimulatory molecule expressed. CTLA-4 – cytotoxic lymphocyte antigen-4; GITR – glucocorticoid-induced TNF-related protein; CD40L – CD40 ligand.

In addition to the above markers, human Tregs also express the transcription factor, Foxp3 (see below), and recent studies have found that additional markers further distinguish Treg populations in humans. Studies have shown that non-regulatory T cells are IL-2 independent, but IL-7-dependent, whereas Tregs are dependent on IL-2 for survival, suggesting that Tregs might not actually require IL-7. This suggested that Tregs can be distinguished based on their high expression of CD25 and low expression of CD127 (68). Analysis of $CD127^-$ and $CD127^+$ Treg populations, not just from peripheral blood, but also thymus, LNs and cord blood, revealed that the $CD127^-$ population are anergic and significantly better suppressors than their $CD127^+$ counterparts (68). Additionally, CD127 expression is negatively correlated with Foxp3 expression: mRNA for Foxp3 is higher in $CD127^-$ Tregs compared to $CD127^+$ Tregs (68, 69).

The α-chain of the integrin $\alpha_E\beta_7$ (CD103) has been shown to be a specific marker of mouse Treg subsets. This allows Tregs to be divided into 3 groups defined by the co-expression of CD25 ($CD103^-CD25^+$, $CD103^+CD25^+$, and $CD103^+CD25^-$), and all three populations have been shown to express similar levels of Foxp3 and are equally suppressive, suggesting that, in this case, CD25 is not necessarily required to define Treg populations (70, 71).

Recently, it has been demonstrated that CD103 can also be used to define human Treg populations. Similar to mouse $CD103^+$ Tregs, division of $CD103^+$ T cells into the above three sub-populations revealed that there is no difference in the suppressive capacity or Foxp3 expression between the groups. Additionally, it was shown that TGF-β is able to induce CD103 expression on $CD4^+$ T cells - since TGF-β can promote CD103 gene transcription (72).

1.1.5 *FOXP3 AS A DEFINITIVE MARKER FOR NATURAL REGULATORY T CELLS*

Although CD25 remains a good marker for identifying Tregs, it is also a marker for activated T cells, and consequently, the argument of whether $CD4^+CD25^+$ T cells are actually Tregs, or just activated T cells, arises. A Treg-specific transcription factor has been identified. Mutations in the gene encoding the protein, Foxp3, both in mice and humans, leads to a severe lymphoproliferative, multi-organ, autoimmune disease.

1.1.5.1 FOXP3 – EVIDENCE FROM SCURFY MICE

The scurfy (*sf*) phenotype results from an X-linked recessive mutation (X^{sf}/Y). Affected male mice externally display scaling of the ears, feet and tail. Internally, the mice suffer from severe lymphadenopathy, splenomegaly, hepatomegaly and lymphocytic infiltration of the skin and liver, ultimately leading to early mortality. These phenotypic abnormalities coincide with overproduction of specific cytokines, which include IL-2, IL-4, IL-6, IL-10 and TNF-α(73). The pathology observed in X^{sf}/Y mice resembles pathology usually seen in autoimmune pathology, thereby suggesting scurf*y* results from some form of defect in central tolerance.

Initial experiments showed that neonatal thymectomy of X^{sf}/Y mice ameliorated disease and prolonged lifespan, but did not prevent disease. X^{sf}/Y mice bred with female nude (congenitally athymic) mice results in a progeny that are athymic with a X^{sf}/Y, *nu/nu* genotype. The male offspring (scurfy-nude) from this progeny do not develop disease. This suggested that T cells are crucial in the development of the disease (74). When bone marrow cells from X^{sf}/Y mice were transplanted into MHC-compatible normal recipients, the donor cells appear in the normal lymphoid compartment of the recipient, but disease does not develop, indicating that the normal thymus is capable of inhibiting the development of pathogenic T cells. However, when thymuses from X^{sf}/Y mice are grafted into nude or SCID mice, the scurfy phenotype prevailed in the recipient (74, 75).

These experiments therefore suggest that scurfy results from defective thymic T cell development. Subsequent investigations showed that the $CD4^+CD8^-$ T cell population are pathogenic and therefore responsible for disease, since adoptive transfer of X^{sf}/Y $CD4^+$ T cells, but not $CD8^+$, into nude mice, results in a scurfy phenotype (76).

Ex vivo $CD4^+$ T cells from scurfy mice have an activated phenotype, expressing increased levels of cell surface activation markers including CD25, CD69, CD80, CD86 and CTLA-4; and decreased levels of CD62L (L-selectin). In addition, analyses of $CD4^+$ T cells from scurfy mice have shown that these cells are highly sensitive to stimulation through their TCR, proliferating approximately 6-times more, compared to control $CD4^+$ T cells (77). Interestingly, the phenotype observed in scurfy mice is similar to mice deficient in CTLA-4 ($CTLA\text{-}4^{-/-}$) (44, 43) or TGF-β ($TGF\text{-}\beta^{-/-}$) (78, 79): $CTLA\text{-}4^{-/-}$die within three weeks of birth. Phenotypically, peripheral T cells from $CTLA\text{-}4^{-/-}$ and $TGF\text{-}\beta^{-/-}$ mice express increased levels of activation markers, including CD69, CD44 and CD25, coupled with massive lymphoproliferation and tissue damage. $TGF\text{-}\beta^{-/-}$ mice are born a normal weight, but by day 20 post-birth, the animals experience severe weight loss together with lymphocytic infiltration of the organs, and subsequent death by three weeks of birth.

The gene responsible for scurfy phenotype, encodes the protein Foxp3 (also known as scurfin), which belongs to the forkhead/winged helix family of transcription factors, which includes Foxp1 and Foxp2. Foxp3 is ~430 amino acids (aa) in length and consists of four functional domains: an amino (NH_2)-terminus repressor domain (aa~75-130), zinc-finger (aa~200-220), leucine-zipper motif (aa~240-260), and a carboxy (COOH)-terminus DNA-binding forkhead (FKH) domain (aa~340-420). Foxp3 differs from other FKH members, because its FKH domain is at the COOH end, but is located in the centre of Foxp1 and Foxp2 proteins. In addition, Foxp3 contains a putative nuclear localisation sequence – arginine, lysine, lysine, arginine (R,K,K,R), within the FKH domain (aa 414-417) (see figure 1.1.4).

Analyses have shown that each of the domains in Foxp3 has a specific function. The FKH domain is important in nuclear import of Foxp3, since targeted mutations of amino acids within the nuclear localisation sequence, inhibits nuclear import (80). In order to function, Foxp3 must homodimerise. Deletion of a glutamic acid residue within the leucine zipper domain, but not in the zinc-finger domain, inhibits homodimersation of Foxp3 (80, 81). The amino terminus of the Foxp3 protein also has a functional domain. Constructs containing aa 1-132 and aa 67-198 of Foxp3 are able repress transcription. This suggested that overlapping sequences between the two constructs are responsible for transcriptional repression.

Additional constructs were developed: aa 1-105, aa 67-132 and aa 106-198. The aa 106-198 construct was shown to be unable to repress transcription, whereas constructs aa 1-105 and aa 67-132, are both equally effective at transcriptional repression. Further investigation showed that aa 67-132 was twice as effective at repressing transcriptional repression than constructs containing aa 1-198 (which incorporates all three constructs mentioned). Therefore, Foxp3 consists of an NH_2-terminus transcriptional repressor domain.

The various functional domains within Foxp3 suggest that the scurfy phenotype arises due to specific mutations within these domains (80). A 2-base pair insertion was found to occur in the coding region of the *Foxp3* gene of scurfy mice (82). Consequently, a frameshift ensues, whereby a premature stop codon is read and the FKH domain is lacking, resulting in a truncated gene product and therefore a non-functional Foxp3 protein. The hyper-lymphoproliferative pathology seen in scurfy mice, suggests that a functional *Foxp3* gene is responsible for regulating peripheral lymphocyte numbers, and studies have demonstrated that over-expression of wild-type (normal) *Foxp3* gene in scurfy mutant mice, prevents disease (83).

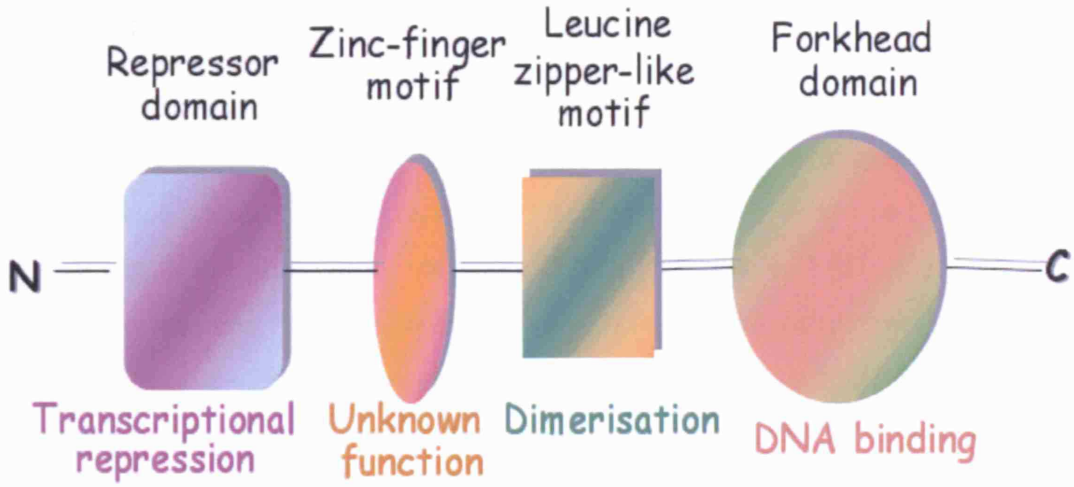


FIGURE 1.1.4 FUNCTIONAL DOMAINS OF THE FOXP3 GENE

Moreover, transgenic mice, which over-express the *Foxp3* gene, have a significantly reduced number of peripheral T cells. In contrast to scurfy $CD4^+$ T cells, transgenic $CD4^+$ T cells are hyporesponsive to TCR stimulation and produce low levels of IL-2. Transgenic T cells were shown to have a hypo-activated phenotype, with higher expression of CD45RB and CD62L, compared to scurfy mutant mice (83). Additionally, T cell development and phenotype within the thymus is not significantly different to scurfy mutant mice. Taken together, these results suggest that Foxp3 exerts its activity in the periphery, where the functional capacity of peripheral T cells is a consequence of the amount of functional Foxp3 protein

The impact Foxp3 has on T lymphocytes suggests that it functions as a negative regulator in peripheral T cell activation. Subsequent studies have shown that Foxp3 is indeed a transcriptional repressor (84). Foxp3, but not Foxp1 or Foxp2 suppresses cytokine production, including IFN-γ, IL-2 and IL-4 (81, 85). By using a reporter gene, it was shown that the presence of a fully functional Foxp3 was capable of repressing transcription. However, if the FKH domain was missing, transcriptional repressor activity of Foxp3 was lost. Scurfy mice have elevated levels of the transcription factors, NFAT (nuclear factor of activated T cells) and NF-κB (Nuclear factor-κB) (85).

NFAT and NF-κB are both transcriptional activators (trans-activators) for cytokine genes including IL-2, IL-4, IL-6 TNF-α and GM-CSF – cytokines that are elevated in scurfy mice. Deletion of the repressor domain abrogated the ability of Foxp3 to suppress the above cytokines. Since Foxp3 exerts its effects on multiple cytokines, this suggests that Foxp3 interferes with the function of trans-activators of cytokine genes, rather than repressing individual cytokine genes (85). Foxp3 has been shown to physically bind to the P65 (RelA) subunit of NF-κB (NF-κB is made up of five subunits: P50, P52, P65, RelB and cRel) (85), and also physically binds to NFAT (85, 81). In both cases, Foxp3 does not interfere with DNA binding of the trans-activators, but instead, acts as a co-repressor, preventing NF-κB and NFAT from binding to their respective promoter regions, which is required for their transcriptional activity.

1.1.5.2 *FOXP3*-EVIDENCE FROM IPEX-AFFECTED HUMANS

Phenotypic similarities between scurfy and its human equivalent, the recessive disorder immune dysregulation, polyendocrinopathy, enteropathy, X-linked, or IPEX, suggested that similar mutations in the *Foxp3* gene may also exist in IPEX patients. Heterozygous female carriers are clinically unaffected. However, heterozygous males suffer from neonatal insulin-dependent diabetes mellitus (IDDM), infections, diarrhoea, gastritis, thryroiditis, cachexia, and usually die within two years of birth. In one study, five families were investigated, all with at least two males with IPEX.

Foxp3 mutations were identified in four out the five families, with all mutations lying within the FKH DNA-binding domain (86). Additional studies carried out in families with affected IPEX male revealed that of the five IPEX males, one male had a point mutation in their *Foxp3* gene, where an alanine was substituted by guanine, within exon 9 – the exon that corresponds to the FKH domain (87). The resultant effect is the same as Foxp3 mutation in scurfy mice, described recently (82): a frameshift, leading to a premature stop codon, and consequently, a non-functional truncated protein product. In the remaining four affected males (all from the same family), and in first and second-generation female carriers, but not in other family members, a different Foxp3 mutation was observed. Here, a 3bp deletion in exon 7 that corresponds to the leucine zipper domain, results in a protein product, which lacks a glutamic acid residue, resulting in impaired Foxp3 homodimerisation, and therefore, loss of functional Foxp3.

1.1.5.3 INDUCED EXPRESSION OF FOXP3

The findings that over-expression of Foxp3 leads to a decrease in peripheral T cell number, in addition to its role as a transcriptional repressor of cytokine genes that are normally involved in autoimmune pathology, suggest that Foxp3 also serves to function as an immune regulator. Support for this view comes from the requirement of Foxp3 in Treg development. The *Foxp3* gene is predominantly transcribed in $CD4^+CD25^+CD8^-$ thymocytes, and similarly peripheral $CD4^+CD25^+$ T cells, but not $CD4^+CD25^-$, $CD8^+$ T cells or B cells (88, 89). These findings provide evidence that Foxp3 is not an activation marker: Foxp3 expression was not induced in T effectors following polyclonal stimulation plus IL-2. Similarly, following polyclonal stimulation (plus IL-2) for 72 hours, Foxp3 levels remain unchanged in Tregs (88), therefore Foxp3 expression is stable, at least in mice, regardless of activation.

Studies have shown that forced expression of Foxp3 in cells that lack Foxp3 expression results in the acquisition of a regulatory phenotype: transduced cells proliferated poorly to TCR-mediated stimulation, produced very little IL-2, and expressed high levels of CD25, GITR and CTLA-4 on their surface, all of which are phenotypically characteristic of Tregs. In addition, these Foxp3-transduced cells were shown to be functionally suppressive both *in vitro* and *in vivo*, where transfer of normal Foxp3-transduced $CD4^+CD25^-$ T cells into SCID mice with induced autoimmune gastritis prevented disease. (88).

Similarly, transgenic mice that over-express Foxp3 have increased $CD4^+CD25^+$ T cells, compared to non-transgenic controls (90). As mentioned above, the phenotype of scurfy mice, resembles the phenotype of mice deficient in CTLA-4 or TGF-β. Over-expression of Foxp3 in CTLA-$4^{-/-}$ mice prolonged survival (up to 180 days, as opposed to just 3 weeks), increased numbers of functionally suppressive Tregs and reduced lymphoproliferation, thereby suggesting that functional Foxp3 can override the defect from lack of CTLA-4 (90).

1.1.6 THYMIC DEVELOPMENT OF REGULATORY T CELLS

Thymic development of Tregs requires specific interactions between their TCRs and self-peptide/MHC complexes expressed on thymic stromal cells $CD4^+CD25^+$ Tregs from TCR transgenic mice predominantly express endogenous TCR α-chain, whereas $CD4^+CD25^-$ T cells from these mice predominantly express both α- and β- chains (91). Confirmation of the expression of the TCR α-chain was seen in RAG2 knockout ($RAG2^{-/-}$) mice. In $RAG2^{-/-}$ mice, Treg development is severely abrogated, suggesting that autoreactive T cells that have high affinity for self-peptide/MHC interactions may undergo receptor editing, and be positively selected to develop into Tregs.

Further support for positive selection as a means for Treg development came from experiments carried out in TS1 transgenic mice. These mice over-express the 6.5 TCR, which is specific for influenza haemagglutinin (HA), which is over-expressed in HA28 transgenic mice. Cross breeding of the two transgenics results in double transgenic TS1 X HA28 mice, whose T cells are autoreactive. These mice have significantly higher numbers of functionally suppressive Tregs than TS1 transgenic mice alone, and express both CD69 and CD5. Development of Tregs in the double transgenics depends on high-affinity and high avidity interactions between TCR and MHC, since transgenic mice with low affinity TCRs for HA, do not develop Tregs (92).

Analysis of the development of Tregs in single TCR transgenic mice has shown that by crossing mice expressing OVA peptide systemically in their nuclei (Ld-nOVA), with OVA-specific TCR transgenics (DO11.10) – resulting in mice with autoreactive T cells that are OVA specific – Treg development is enhanced, with increased numbers present in both the thymus and the spleen. By comparison, DO11.10 mice have significantly fewer Tregs both centrally and peripherally. Analysis of endogenous TCR chains on Tregs revealed that double transgenics express significantly lower levels than DO11.10 mice.

To determine whether Treg development in the double transgenics is a consequence of positive selection, Ld-nOVA mice were crossed with DO11.10 α-chain transgenic mice. All T cells from the double transgenics develop into Tregs, whereas no Tregs developed in DO11.10 α-chain transgenic mice (93). These results therefore suggest that intrathymic development of Tregs relies on (some) autoreactive T cells that go through positive selection, via endogenous TCR α-chain expression.

Analysis of Tregs from DO11.10 mice have shown that a high proportion of these cells express endogenous TCR β-chains, at similar levels seen in double transgenic $CD4^+CD25^-$ cells. $RAG2^{-/-}$ DO11.10 mice completely lack Tregs, whereas Ld-nOVA-$RAG2^{-/-}$ transgenics still develop Tregs. These results suggest that TCR rearrangement must occur in non-autoreactive T cells, firstly to escape negative selection, and secondly to form auto-specific TCRs that have an appropriate affinity to MHC that will lead to Treg development - thymic development of Tregs is therefore unique, in that positive selection of these cells relies on correctly balanced high-affinity, high-avidity TCR-self peptide/MHC II interactions, so that Tregs are not deleted.

Thymic epithelial cells TECs have been shown to be important in Treg development. Transplantation of peripheral skin tissue grafts into allogeneic recipients lead to graft rejection. However, if skin is grafted into an allogeneic recipient, together with TECs from the same donor, tolerance of the graft is induced (94). These early experiments, however, did not define which TECs were responsible for inducing tolerance. Evidence for the importance of cTECs and mTECs in Treg development has been demonstrated. Both CD25 expression and Foxp3 expression occurs at the $CD4^+$SP stage in developing thymocytes (95), suggesting that Treg development occurs in the thymic medulla.

Investigation into the kinetics of Foxp3$^+$ Treg development within the thymus utilised Foxp3 knock-in mice, where the *Foxp3^{gfp}* allele encodes a Foxp3-GFP fusion protein. This allows the investigator to physically visualise locations where Foxp3 is expressed within the thymus. Initial experiments in this study found that just 12 hours postnatal, 4% of CD4$^+$SP thymocytes express CD25. However, when the kinetics of Foxp3 expression was analysed on the same subset of cells, it takes approximately 21 days for Foxp3 expression to reach the same levels as CD25. By immunohistochemical staining, the authors showed that Foxp3$^+$ cells at day 21 postnatal are almost exclusively localised to the thymic medulla, with very few Foxp3$^+$ cells present at the cortico-medullary junction (96).

Efficient thymic Treg development relies on CD28 co-stimulation. CD28$^{-/-}$ mice were shown to have significantly lower levels of CD4$^+$CD25$^+$ T cells, coupled with abrogated suppressive activity of these cells. Moreover, co-engagement of TCR and CD28 in *in vitro* cultures containing CD4$^+$CD8$^+$DP thymocytes resulted in Foxp3 induction (97). Complementary to these results, it has been demonstrated that CD86 expression is restricted to the thymic medulla, suggesting mTECs consist of APCs that are capable of providing sufficient TCR-self-peptide MHC interactions, necessary for Treg development.

Further evidence for mTECs behaving as APCs during Treg development comes from very recent studies, in which chimeric mice were generated whereby only TECs express MHC II. By adding equal numbers of CD45$^-$ thymic cells (therefore eliminating haematopoietic cells) from wild-type mice and MHC II deficient (*H2-Ab1$^{-/-}$*) mice into *H2-Ab1$^{-/-}$* recipients, it was possible to identify distinct cortical and medullary regions that were both MHC II negative and positive. Further analysis of the regions showed that only the MHC II positive regions in the medulla, but not cortex, consist of Foxp3$^+$ Tregs (98). Moreover, mTEC-derived antigen is responsible for Treg differentiation.

Evidence supporting the thymic medulla as the site for Treg selection comes from experiments looking at factors produced by Hassall's corpuscles. These epithelial cell structures are situated in the medulla and are well developed in humans and guinea pigs, but poorly developed in the murine thymus. They produce a variety of cytokines including TGF-α, and an IL-7-like cytokine, thymus stromal lymphopoietin (TSLP).

Murine (m) TSLP and IL-7 are functionally similar. Both cytokines stimulate developing thymocytes, as well as mature T cells. The mTSLP receptor is a heterodimeric complex, where it binds sequentially; first binding with low affinity to TSLPR, followed by a high affinity complex with IL-7Rα (99). The binding of cytokines to their receptor, for example IL-2, leads to the activation of the Janus kinase (Jak)/Stat pathway, where Jak1 binds to the IL-2Rβ chain, and Jak3 binds to γc and the net effect is phosphorylation of the Stat5 transcription factor.

Human (h) TSLP also binds to a heterodimeric receptor complex. hTSLP comprises of the IL-7Rα chain and TSLPR, both of which are co-expressed on $CD11c^+$ myeloid DCs. Binding of hTSLP to its receptor on DCs up-regulates surface expression of the co-stimulatory molecules CD40 and CD80, thereby inducing DC activation (100).

TSLP-induced activation of medullary $CD11c^+$ DCs induces Tregs in the thymus. By isolating T cell lineage thymocytes, the authors showed that culture of these cells with $CD11c^+$ DCs stimulated with hTSLP, but not IL-7 or CD40 and induces the expansion of $CD4^+CD8^-CD25^+$ T cells, which also express the Treg transcription factor, Foxp3 (101). In addition, these TSLP-induced Tregs are functionally suppressive, inhibiting $CD4^+CD25^-$ T cell proliferation, a characteristic feature of Tregs.

In contrast, however, some studies have shown that thymic Treg development is dependent on MHC II expression on cTECs. By generating transgenic mice, whereby negative selection, and hence clonal deletion is lost, $K14_{\beta}^{b}$ mice (where MHC II is solely expressed on cTECs, whereas mTECs and other BM-derived APCs are MHC II negative) were shown to still develop anergic and functionally suppressive Tregs at levels similar to that seen in wild-type mice (102).

The ontogeny of Tregs during thymic development is controlled by the cytokines critical for T-cell development that are produced by thymic epithelial cells, stromal cells and DCs. IL-7 has been shown to be required for the development, homeostatic proliferation and maintenance of T cells. The receptor specific for IL-7, IL-7Rα, is highly expressed in thymocytes as well as in mature naïve T cells.

Following acute activation, IL-7Rα is down-regulated (103). IL-7 signalling promotes the survival and proliferation of thymocytes and mature peripheral T cells, but it is suppressed when T cells receive survival signals from other cytokines, such as IL-2, IL-4, IL-7 and IL-15 (104). Since Tregs constitutively express CD25 (IL-2Rα) and are able to out-compete for IL-2 binding over naïve T cells to receive IL-2-mediated survival signals (105), IL-7 signalling is down-regulated in Tregs, and this is supported by evidence that shows Tregs express lower levels of IL-7Rα, which results in their decreased ability to proliferate under lymphopenic conditions. These findings therefore question whether IL-7 signalling is directly involved in the generation and maintenance of Tregs.

1.1.7 MIGRATION OF THYMIC REGULATORY T CELLS IN THE PERIPHERY

The final developmental stage of Tregs (as well as conventional T cells) is the surface expression of CD62L. Therefore, thymically derived Tregs leave the thymus with a $CD4^{+}CD25^{+}Foxp3^{+}CD62L^{+}$ phenotype. Murine studies have shown $CD62L^{+}$ Tregs are better functional suppressors in terms of inhibiting diabetes in NOD mice, than their $CD62L^{-}$ counterparts (106). The $CD62L^{+}$ subset of Tregs has also been shown to be better at preventing graft vs host disease (GvHD) (107).

This difference in *in vivo* suppressive activity may be due to the homing capacity of the two subsets of Tregs: $CD62L^{+}$ Tregs are able to efficiently migrate to secondary lymphoid organs, and encounter subsequent priming processes, much better than $CD62L^{-}$ Tregs. Evidence for this comes from experiments where administration of neutralising antibody to CD62L results in allograft rejection, due to the prevention in Treg expansion in the draining LNs (108).

Tregs in human secondary lymphoid organs that have just left the thymus express CCR7. This enables them to migrate to the T cell zones, which express the CCR7 ligand, CCL19. However, upon T cell priming, the Treg migratory response to CCL19 reduces, due to the rapid induction of the chemokine receptor CXCR5. This results in a change in migration of the Treg towards the CXCR5 ligand, CCL13, which is expressed in the B cell zone of secondary lymphoid organs, which enables them to suppress germinal centre (GC)-T helper (Th) cells and GC-Th cell-induced B cell responses such as B cell activation and Ig production (109).

Tregs in human peripheral blood express the chemokine receptors CCR4 (and CCR8). These chemokine receptors are preferentially expressed by Th2 cells, as well as newly primed T cells, but not naïve T cells. The ligands for CCR4 - CCL17 and CCL22 - are expressed on mature DCs, which enables them to attract Tregs from the peripheral blood to secondary lymphoid organs, as well as sites where they can be primed for suppressor functions (110) (see figure 1.1.7).

In addition to chemokine receptors, integrins regulate Treg migration to sites of inflammation. For example, Tregs that are $CD103^{+}$ (the α_E subunit), express low levels of CCR7 and CD62L and migrate into and suppress peripheral inflammation; whereas $CD103^{-}$ express high levels of CCR7 and CD62L, which facilitates efficient migration into LNs and suppression of naïve T cell activation (70, 71), suggesting that expression of specific homing molecules dictates where (anatomically) Tregs are functionally suppressive (see figure 1.1.7).

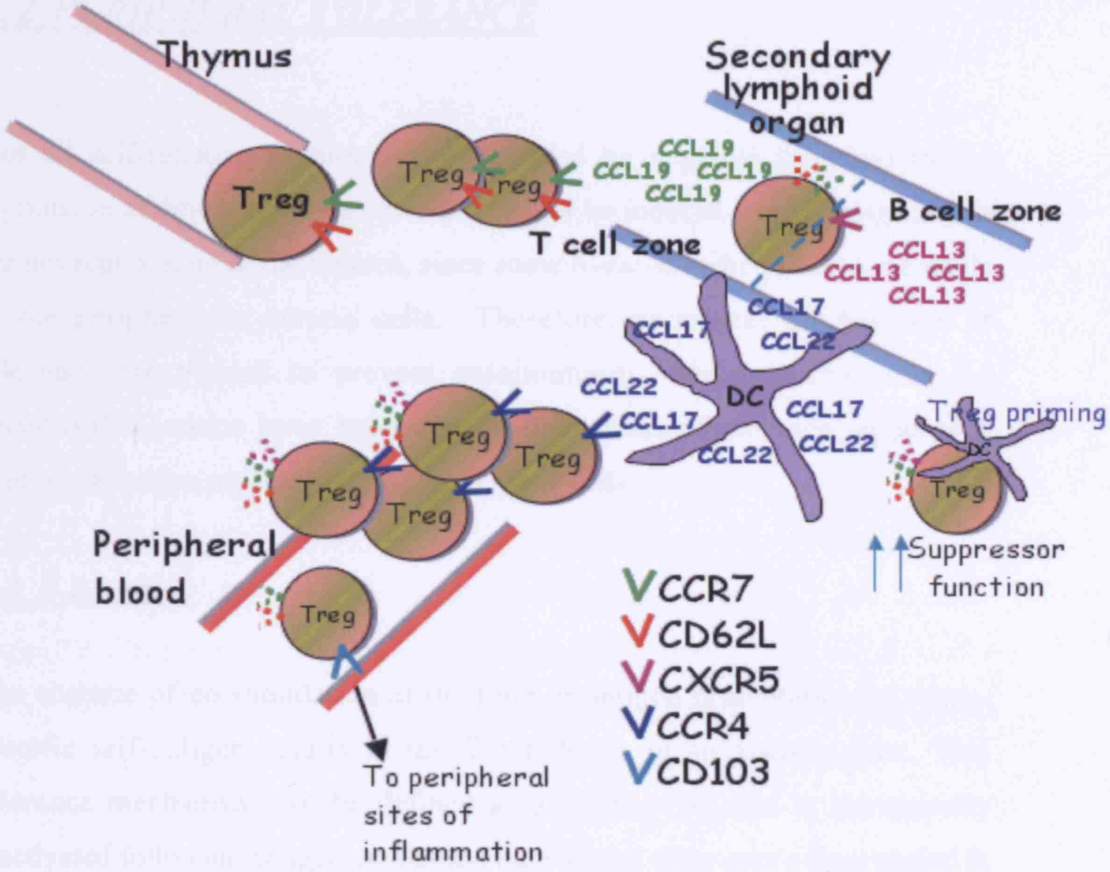


FIGURE 1.1.7 MIGRATION OF REGULATORY T CELLS IN THE PERIPHERY

Tregs express different chemokine receptors and homing molecules on their surface depending on a) their anatomical origin; b) the up-regulation of specific chemokines and ligands; and c) their activation status. Following their development in the thymus, Tregs, like their naïve T cell counter parts, express CD62L and CCR7, enabling them to migrate to the secondary lymphoid organs. Within the lymphoid organs, Tregs express differential chemokine receptors, depending on whether they need to migrate to T cell or B cell zones. Circulating Tregs in the peripheral blood up-regulate CCR4 on their surface, enabling them to be primed for suppressive function via mature DCs.

1.2 PERIPHERAL TOLERANCE

Not all self-reactive thymocytes are deleted by negative selection in the thymus. In addition, negative selection cannot be induced to self-antigens that are not represented in the thymus, since some tissue-specific proteins are made in the periphery by certain cells. Therefore, peripheral mechanisms of tolerance are needed to prevent autoimmunity. Many mechanisms for peripheral tolerance have been described, including ignorance, apoptosis, anergy and active suppression by regulatory T cells.

1.2.1 ANERGY

The absence of co-stimulation at the time of antigen presentation of tissue-specific self-antigen results in the T cell being in an anergic state. This tolerance mechanism can be defined as a lymphocyte that is intrinsically inactivated following antigen encounter, but remains alive over a long period in a hyporesponsive state. Therefore, anergic T cells do not proliferate, nor do they produce cytokines, such as the T cell growth factor IL-2.

Experiments carried out originally by Jenkins and Schwartz demonstrated that antigen-specific T cells that are cultured with chemically treated APCs fail to undergo proliferation. Moreover, re-stimulation of these T cells with normal APCs renders the T cells unresponsive, both *in vitro* and *in vivo*. The chemically treated APCs used in these experiments still had functional Ia molecules and therefore MHC-peptide presentation was not affected, suggesting that the absence of other signals are responsible for T cell unresponsiveness (111).

This form of antigen-specific T cell unresponsiveness is referred to as clonal T cell anergy. Subsequent experiments carried out by the same authors demonstrated that Th1 clones that are initially stimulated *in vitro* with fixed APCs, and then rechallenged with anti-CD3 antibody without anti-CD28 co-stimulation, become hyporesponsive, where they fail to proliferate and produce IL-2, providing evidence that the absence of co-stimulation, or signal 2, is required for T cell anergy. However, the addition of exogenous IL-2 is able to overcome the anergic state (112).

The above *in vitro* experiments demonstrate the importance of "signal 1" in the induction of anergy. Further *in vitro* experiments have also established the importance of signals generated through TCR engagement in T cell anergy. Altered peptide ligands (APLs) are modified peptide agonists that still have the ability to engage TCR, but are unable to induce co-stimulation, and hence full T cell activation. In addition, engagement of APL even in the presence of co-stimulation or subsequent stimulation with the same, but unaltered, agonistic peptide, fails to inhibit T cell anergy (113). Other *in vitro* mechanisms that induce anergy include the addition of low doses of agonistic peptide (114, 115).

These and the above experiments suggest that suboptimal activation, either in the absence of co-stimulation, or inadequate TCR engagement, even in the presence of co-stimulation, is sufficient to induce clonal T cell anergy. Experiments carried out *in vivo* have also provided evidence for suboptimal activation in the induction of clonal T cell anergy, and have demonstrated the requirement of pre-activated T cells to promote peripheral T cell tolerance: T cell must reach their effector phase (i.e. proliferate and produce cytokines, as well as the induction of inflammatory autoimmune pathology in an organ expressing the cognate antigen), before tolerance can be induced (116).

Similarly, naïve T cells that are subjected to strong TCR stimulation, in the absence of co-stimulation, are unable to become anergic. Instead, these T cells become more responsive to CD3/antigen stimulation, and resemble memory T cells both in terms of phenotype ($CD69^+$ $CD62L^-$) and their kinetics (117). Findings from both these studies therefore suggest that induction of immunogenic and tolerogenic/anergic T cell differentiation are similar, since both rely on the development of effector function, and negative signalling through TCR stimulation is regulated by the development of $CD4^+$ T cells.

The experiments, described above also showed that the induction of T cell anergy is inhibited by the addition of cyclosporine A (CsA) (112). CsA is an inhibitor of the phosphatase calcineurin, which facilitates translocation of the Ca^{2+}-regulated transcription factor, nuclear factor of activated T cells (NF-AT) into the nucleus. Once in the nucleus, NF-AT associates with another transcription factor, AP-1, and both go onto regulate the transcription of various cytokine genes, including IL-2 and IL-4. Inhibition by CsA therefore suggests a mechanism by which anergy is elicited.

Th1 cells derived from NF-$AT^{-/-}$ mice are resistant to CD3-mediated induction of anergy. More specifically, T cells that are transfected with a constitutively active (CA) form of NF-AT (which associates normally with AP-1) are unable to undergo anergy. In contrast, T cells that are transfected with an engineered derivative of CA NF-AT, which cannot associate with AP-1, become anergic due to the up-regulation of anergy-associated genes, such as Cbl-b (118). Consequently, inhibition of NF-AT-AP-1 associations in the nucleus promotes T cell anergy.

Further investigation into the mechanism(s) that induce anergy have shown that it is dependent on the cell-cycle phase. Addition of the immunosuppressive agent rapamycin - which arrests the cell cycle at the late G1 phase - to T cells induces anergy, even in the presence of co-stimulation and IL-2 production.

However, if the cell cycle is arrested at early S phase (by the addition of hydroxyurea), anergy does not develop (119). Results from these experiments therefore infer that the induction of clonal T cell anergy is not necessarily dependent on the absence of co-stimulation and/or IL-2, but on changes that take place during the cell cycle between G1 and S phase.

1.2.2 PERIPHERAL TOLERANCE INDUCED BY REGULATORY T CELLS

The efficient migration of natural Tregs (i.e. $CD4^+CD25^{hi}Foxp3^+$) into the periphery, following their development in the thymus, allows them to suppress potentially pathogenic, self-reacting T cells ($CD4^+$, as well as $CD8^+$) and promote peripheral tolerance. Evidence for the mechanisms by which natural Tregs function originally came from *in vitro* experiments, suggesting that these cells suppress in a mainly cell-contact dependent, cytokine-independent fashion, mediated by surface molecules such as CTLA-4 and GITR.

Evidence for this comes from initial studies, which demonstrated that in the presence of a semi-permeable membrane, Treg suppression is abrogated (19). Similarly, supernatants collected from cultured Tregs are unable to suppress $CD4^+$CD25- T cells (20). However, increasing evidence suggests that natural Tregs can also function through specific cytokines, such as TGF-β. Moreover, numerous studies suggest that Tregs can develop in the periphery from naïve T cells, such as Tr1 cells (120) , or be induced following oral tolerance – Th3 cells (121).

1.2.2.1 TGF-β IN REGULATORY T CELL DEVELOPMENT AND FUNCTION

TGF-β SIGNALLING PATHWAY

The TGF-β family of cytokines comprises of at least four different protein members, including bone morophogenic proteins, growth differentiation factors and TGF-βs. Within the TGF-β group, there are 3 members, namely TGF-β1, TGF-β2 and TGF-β3. TGF-β1 (referred to hereafter as TGF-β) is the isoform that is commonly associated with, and predominantly expressed during immune responses.

Biological activity of TGF-β is regulated at the post-translational level, which involves a series of co-ordinated responses that serves to release newly synthesised TGF-β from its inhibitory protein complex. Newly synthesised TGF-β exists in a latent/inactive form and is non-covalently associated with a latency-associated peptide (LAP), forming a latent complex. LAP prevents TGF-β from interacting with its receptors, which are expressed on the surface of its target cells, and therefore TGF-β needs to be cleaved from the complex in order to exert its effects. This is achieved, for example, through binding of the integrin fibronectin ($\alpha_v\beta_6$) (122). The main function TGF-β is to inhibit T cell proliferation, and does so by inhibiting IL-2 gene expression, thereby blocking IL-2 production (123)

TGF-β binds to type I and II transmembrane serine/threonine receptors, which comprise of five type I members and seven type II members. TGF-β dimerizes and binds to the type I receptor, (TGF-βRI; ALK5) and TGF-β receptor II (TGF-βRII), which initiates the signalling cascade (see figure 1.2.2.1). Binding of TGF-β triggers TGF-βRII association with, and subsequent phosphorylation and activation of TGF-βR1. TGF-β signalling is regulated by intracellular Smad proteins, which comprise of four receptor Smads (R-Smad 1, 2, 3 and 5), one common Smad (co-Smad 4), and two inhibitory Smads (I-Smad 6 and 7).

Phosphorylation of TGF-βR1 induces phosphorylation of Smad 2 and 3, which subsequently associate with co-Smad 4, which facilitates translocation into the nucleus and binding to the Smad binding element. The I-Smads inhibit TGF-β signalling and are regulated by TGF-β itself in a negative feedback loop. Smad 7 inhibits TGF-β signalling in one of three ways:

1. Binding to activated TGF-β receptor, which inhibits activation of Smad 2 and 3.
2. Preventing Smad 2 and 3 associating with co-Smad 4, which prevents translocation to the nucleus.
3. Recruiting Smad ubiquitin regulatory factors (Smurfs) that induce polyubiquitination and subsequent degradation of TGF-βRI.

Recent studies have demonstrated that IFN-γ inhibits Smad 3 phosphorylation (124) and that Th1 cells up-regulate Smad 7 (125).

TGF-β-MEDIATED DEVELOPMENT AND FUNCTION OF REGULATORY T CELLS IN THE PERIPHERY

Increasing evidence suggests that $CD4^+CD25^{hi}Foxp3^+$ Tregs, which are normally considered as thymically derived, are also induced in the periphery. Moreover, the induction of these conventional Tregs appears to involve TGF-β. Initial studies have shown that TGF-β is necessary for the induction of human $CD8^+$ T suppressor cells, demonstrating that $CD8^+$ T cells induce natural killer (NK) cells to produce TGF-β, which in turn induces $CD8^+$ T cells to become suppressive. Moreover, they were able to by-pass the requirement for NK cells, by adding recombinant TGF-β to *in vitro* $CD8^+$ T cell cultures and directly induce suppressor function in these cells (126).

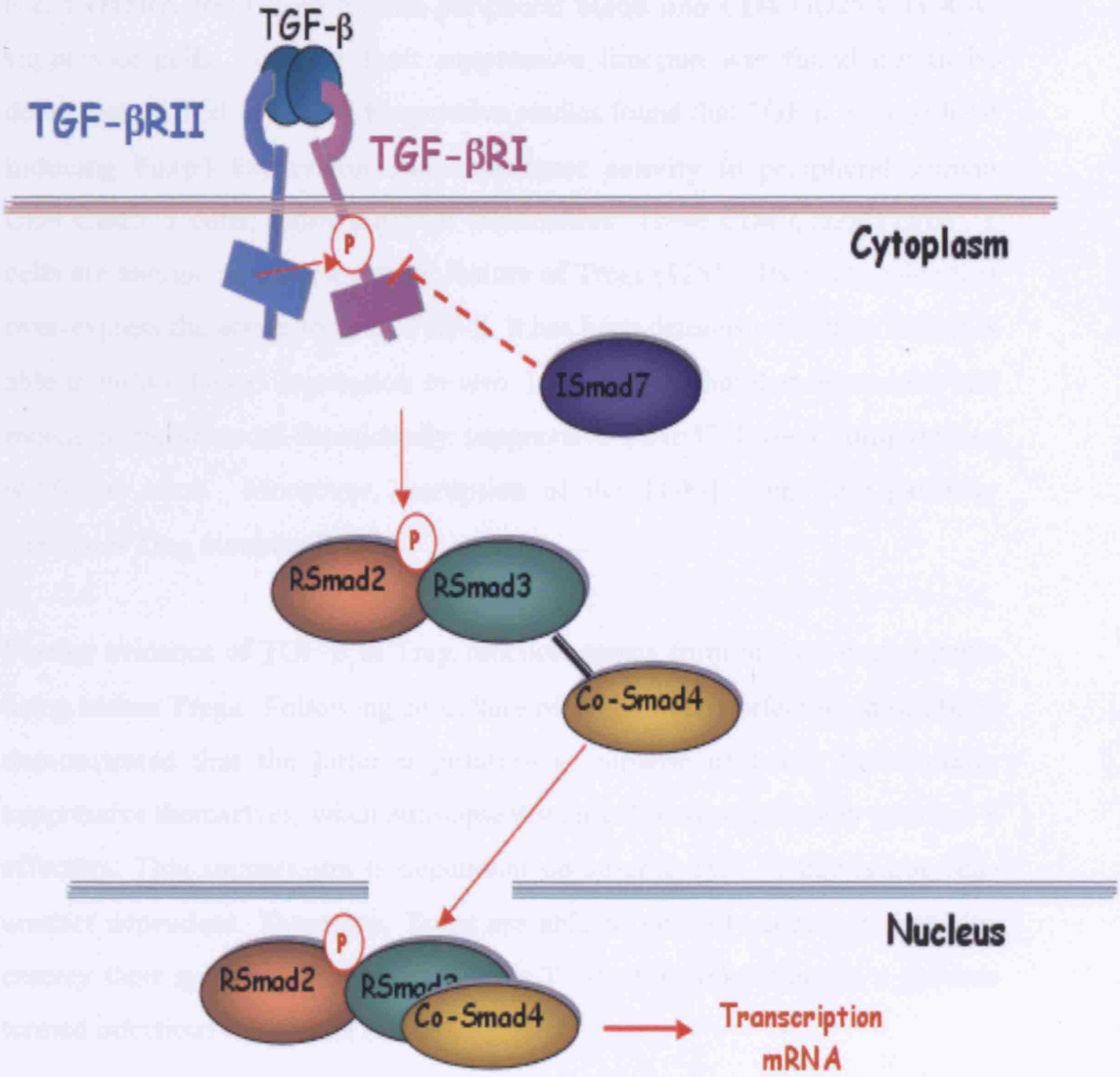


FIGURE 1.2.2.1 THE TGF-β SIGNALLING PATHWAY

Binding of TGF-β to its surface receptors leads to the activation of a Smad-dependent signalling pathway. Initial binding triggers TGF-β receptor II (TGF-βRII) to phosphorylate TGF-βRI. Consequently, TGF-β-specific receptor signalling molecules, Smad 2 and 3 are phosphorylated, which enables them to associate with Co-Smad 4, facilitating their translocation to the nucleus. TGF-β-mediated signalling is an auto-feedback loop, down-regulating signals via inhibitory Smad 7.

Subsequent studies found that TGF-β is able convert naïve $CD4^+$ T cells (i.e. $CD45RA^+RO^-$) from human peripheral blood into $CD4^+CD25^+CTLA\text{-}4^+$ suppressor cells; however their suppressive function was found not to be dependent on TGF-β (127). Progressive studies found that TGF-β is capable of inducing Foxp3 expression and suppressor activity in peripheral human $CD4^+CD25^-$ T cells, following TCR stimulation. These $CD4^+CD25^+Foxp3^+$ T cells are anergic *in vitro*, a classic feature of Tregs (128). By using mice that over-express the active form of TGF-β, it has been demonstrated that TGF-β is able to induce Foxp3 expression *in vivo*. It was found that that these mice had increased numbers of functionally suppressive $Foxp3^+$ T regs compared to wild-type mice. Moreover, disruption of the TGF-β signalling pathway attenuates Treg numbers (129).

Further evidence of TGF-β in Treg function comes from *in vitro* experiments using human Tregs. Following co-culture of Tregs with T effectors, it has been demonstrated that the latter population is capable of being functionally suppressive themselves, when subsequently co-cultured with freshly isolated T effectors. This suppression is dependent on soluble TGF-β, but is not cell-contact dependent. Therefore, Tregs are able to not only suppress, but also convey their suppressive function to the T effector population, in a process termed infectious tolerance (130).

In vitro experiments have shown that TGF-β, in addition to its importance in Treg development, is also important in Treg function: splenic Tregs ($CD4^+CD25^+$) from 8 week-old Balb/c mice constitutively express TGF-β in its inactive form, following TCR stimulation, and the addition of neutralising antibodies to TGF-β abrogates Treg suppressor activity (131). Moreover, responder/effector ($CD4^+CD25^-$) T cells constitutively express TGF-βRII on their surface, suggesting that Tregs function in a cell contact-dependent fashion, mediated by TGF-β (128).

Various investigations into the exact mechanism(s) of how TGF-β exerts its suppressive actions have suggested a role for CTLA-4. Cbl-b, which is critical for T-cell anergy (see above) and is tightly regulated by CD28 and CTLA-4, has also been implicated in Foxp3 expression and $CD4^+CD25^+$ Tregs. The direct evidence for the involvement of Cbl-b in Foxp3 expression is provided by showing that Cbl-$b^{-/-}$ effector T cells did not respond to TGF-β-mediated induction of Foxp3 expression and the conversion into Treg cells (132). In this regard, engagement of CTLA-4 by CD80/CD86 delivers specific signals to Cbl-b which then cooperates with TGF-β signalling molecules to initiate the Smad2/3 phosphorylation, and subsequently the Smad2/3/4 signalling complex (133).

In contrast, TGF-β-induced Foxp3 can directly bind to the inhibitory Smad7 promoter region to turn off its expression, thereby forming a feedback regulation of TGF-β signalling, which may result in the accumulation of Foxp3 expression, facilitating the conversion into $CD4^+CD25^+$ Tregs (125). However, some reports have shown that TGF-$\beta^{-/-}$ (134) or $Smad3^{-/-}$ mice still have functional $CD4^+CD25^+Foxp3^+$ Tregs, which suggests that TGF-β may not be necessary for Foxp3 expression during Treg development in the thymus, but is necessary for Foxp3 expression during Treg development in the periphery.

TGF-β alone is not able to induce Foxp3 expression and IL-2 has been shown to be required for this process: $IL\text{-}2^-CD4^+CD25^-$ T cells fail to express Foxp3 and to be converted into $CD4^+CD25^+$ Tregs in the presence of TGF-β. Additionally, neutralization of IL-2 abolishes TGF-β-mediated induction of immunosuppressive activity (135). Recent evidence suggests a possible reason for the requirement of IL-2 and TGF-β in Treg generation. TGF-β co-operates with TCR signalling to increase CD25 expression, which is mediated by the Smad3/4 complex (136). Therefore, TGF-β relies on IL-2 by promoting the expression of functional high affinity IL-2 receptors.

ORAL TOLERANCE AND THE INDUCTION OF TGF-β-SECRETING TH3 REGULATORY T CELLS

Oral tolerance can be defined as the active suppression of either cell-mediated and/or humoral responses to antigen that has been previously administered by the oral route. Classically oral tolerance is induced when the antigen encounters the gut-associated lymphoid tissue (GALT), and can take place at the GALT or following absorption. Consequently, oral tolerance and mucosal immunity are part of one immune response. GALT is an established immune network, comprising of Peyer's patches (lymph nodes within the gut), villi containing epithelial cells, intraepithelial lymphocytes (IELs), as well as lymphocytes distributed throughout the lamina propria.

The GALT also contain a significant number of APCs, including macrophages, B cells and dendritic cells, with the latter being the major intestinal APC that can acquire and process orally-administered antigen (137) and DCs present in Peyer's patches preferentially induce undifferentiated T helper cell clones (Th0) to produce high levels of IL-4 (138), as well as high levels of IL-10 (139). Therefore, the intestinal mucosa consists of high levels of IL-4, IL-10, as well as TGF-β, making it a perfect cytokine milieu for the development of Th2 and TGF-β-secreting regulatory Th3 cells.

The outcome of oral tolerance depends on two factors: the dose and nature of antigen. Generally, it is considered that administration of high-dose antigen results in clonal anergy (140). Low-dose antigen, on the other hand, results in active suppression characterised by suppression of autoimmune disease and generation of regulatory cells that secrete TGF-β. Oral tolerance in animals fed with low-dose antigen leads to antigen presentation by APCs in Peyer's patches, which preferentially induce the generation of regulatory/suppressor T cells.

Initial experiments using the Lewis rat model of experimental autoimmune encephalomyelitis (EAE; animal model of multiple sclerosis), demonstrated that following oral exposure of these animals to low-dose guinea pig myelin basic protein (MBP), $CD8^+$ T cells isolated from these animals (from spleen and LNs of the gut), are able to suppress EAE both *in vivo* (by adoptive transfer), and *in vitro* (141). Subsequent studies found that TGF-β is responsible for the suppressor function of these $CD8^+$ T cells, since neutralising antibodies to TGF-β abrogates their suppressive capabilities (142).

Further analysis into the induction of suppressor T cells, following oral administration of antigen, found that these cells are also present in murine models of EAE. Moreover, the suppressor T cells were found to be $CD4^+$ as well as $CD8^+$, with an increase in the ratio of T cells secreting IL-4, IL-10 and TGF-β to T cells secreting IFNγ. More specifically, analysis of T cells secreting IL-4 and IL-10 were identified as being produced by the same T cell clone, i.e. Th2 T cell clones, suggesting that TGF-β -secreting T cells are a distinct and separate T cell clone, namely Th3 cells (121, 143).

TGF-β AND THE INDUCTION OF TH17 CELLS

The IL-17 cytokine family consists of six members, namely IL-17A-F, and of the six members, IL-17A is the best characterised and is primarily secreted by $CD4^+$ T helper cells. Originally, it was believed that Th1 cells were responsible for IL-17 secretion; however, it is now clear, at least in the mouse, that a distinct subset of IL-17-producing T helper cells exists, called Th17 cells. Three separate studies have demonstrated that IL-17-producing T cells do not express the Th1 and Th2 transcription factors, T-bet and GATA-3, thereby confirming that Th17 cells exist as a separate T helper subset (144-146). Moreover, the development of Th17 cells requires co-stimulation from both CD28 and ICOS (146).

The induction of Th17 cells from naïve T cell precursors relies on the presence of IL-6 and endogenously produced TGFβ (145). In addition, Th17 and Treg cells are believed to arise from a common T cell precursor, but the development of either subset is dependent on the inflammatory environment: in the presence of TGF-β alone, $CD4^+CD25^-$ T cells are converted to Tregs; however, in the presence of TGF-β *and* IL-6, $CD4^+CD25^-$ T cells are converted to Th17 cells. They also showed that the lack of Treg development in the presence of IL-6 is attributed to the cytokine down-regulating Foxp3 expression, through its promotion of IL-17 production (147) (see figure 1.2.2)

The relationship between TGF-β and peripheral Treg generation suggests that Treg and Th17 development is entirely dependent on the inflammatory environment. Therefore, the environment that TGF-β is exposed to (i.e. in the presence or absence of inflammation) greatly determines the immune response mediated by TGF-β. The importance of TGF-β in Th17 induction is demonstrated by the fact that Th17 cells lack the inhibitory Smad, Smad 7 (145)

Expression of the orphan nuclear receptor, RORγT is required in lymphoid-inducer T cells (LTi), which are required for the development of lymphoid structures, such as Peyer's patches, in the lamina propria. ROR-γT has recently been identified as a Th17-specific transcription factor. They showed that ROR-γT expression is induced upon exposure to TGF-β and IL-6 and is expressed in T cells situated in the lamina propria, the majority of which produce IL-17 constitutively (148).

Th17 cells play an important role in the development of inflammation and autoimmune disease, including EAE and rheumatoid arthritis (149). Moreover, the addition of neutralising antibodies to IL-17 in mice that have collagen-induced arthritis, reduces joint inflammation (150). As well as inducing autoimmune disease, Th17 cells are important in host defence. Il-17 stimulates the generation and mobilisation of neutrophils by up-regulating granulocyte-colony stimulating factor (G-CSF).

Additionally, IL-17 is important in defence against extracellular bacteria, as well as fungal pathogens. The cytokine IL-23 is a member of the IL-12 family, and is a heterodimer comprising of a p19 subunit and the IL-12 p40 subunit, with the latter subunit binding to the IL-12 receptor β1 chain. Initially, it was considered that IL-23 is involved in the induction of Th17 cells, since mice lacking the p19 subunit of IL-23 were found to have very few IL-17-producing cells and do not develop EAE or collagen-induced arthritis (149). However, subsequent studies found that IL-23 is insufficient to induce Th17 development from naïve T cells (151), which were confirmed by findings that functional Th17 cells are still induced from naïve T cells in the presence of IL-23 neutralising antibodies, as long as the cells are exposed to TGF-β and IL-6 (145).

However, despite the lack of role played by IL-23 in Th17 development, the maintenance of Th17 cell effector functions is dependent on IL-23. For example, Th17 cells develop in wild-type mice and mice deficient for IL-23; however, infection with the intestinal bacterial pathogen *Citrobacter rodentium* is cleared in wild-type mice, but not IL-23 deficient mice, suggesting that IL-23 is required for Th17 effector function of host defence (152). In addition to IL-17, Th17 cells secrete IL-22, a cytokine that is important in innate skin infections, and its production is up regulated by IL-23 (153).

In vitro culture experiments have shown that the addition of IFN-γ or IL-4 potently inhibits the development of Th17 cells, but does not inhibit mature Th17 cells (144). This was confirmed in cultures containing $CD4^+$ T cells that are deficient in IFN-γ, who can secrete IL-17, until the addition of exogenous IFN-γ (154). Subsequent experiments have demonstrated that $CD4^+$ T cells deficient in STAT1 and STAT6, the two transcription factors implicated in IFN-γ (Th1) and IL-4 (Th2) production, respectively, exhibited augmented IL-17 production (151).

Taken together, these experiments suggest that Th1 and Th2 responses negatively regulate Th17 cells. By contrast, the addition of exogenous of IL-17 to T cell cultures cannot suppress Th1 or Th2 polarisation, inferring that Th17 cells cannot potentiate their own differentiation by inhibiting Th1 or Th2 differentiation (155). In addition to IFN-γ and IL-4, another cytokine, IL-27 negatively regulates Th17 development. Like IL-23, IL-27 is a member of the IL-12 family, and is related to the IL-6 family, as a component of the IL-27 receptor is made up of the IL-6 receptor subunit, gp130 (the IL-27-specific receptor subunit is WSX-1). Following receptor engagement, both STAT1 and STAT3 are activated.

By using IL-27$^{-/-}$ mice that lack the WSX-1 subunit, it was shown that upon induction of EAE, these animals have augmented neuroinflammation, which correlates with increased numbers of IL-17-producing T cells, demonstrating that IL-27 negatively regulates Th17. More specifically, IL-27 regulation of Th17 cells is dependent on STAT1 (156). Recently, it has been demonstrated that IL-2 inhibits development of Th17 cells. The addition of exogenous IL-2 to naïve T cell cultures containing TGF-β and IL-6 reduces the proportion of Th17 cells; whereas, blockade of endogenous IL-2 enhances Th17 development. Moreover, activation of STAT 5 by IL-2 inhibits ROR-γT expression (157). These results suggest that IL-2 also influences the Treg/Th17 divergence: by inhibiting Th17 development, IL-2 allows the progression of Treg development, by activating STAT5, which has recently been shown to promote Foxp3 expression (158, 159).

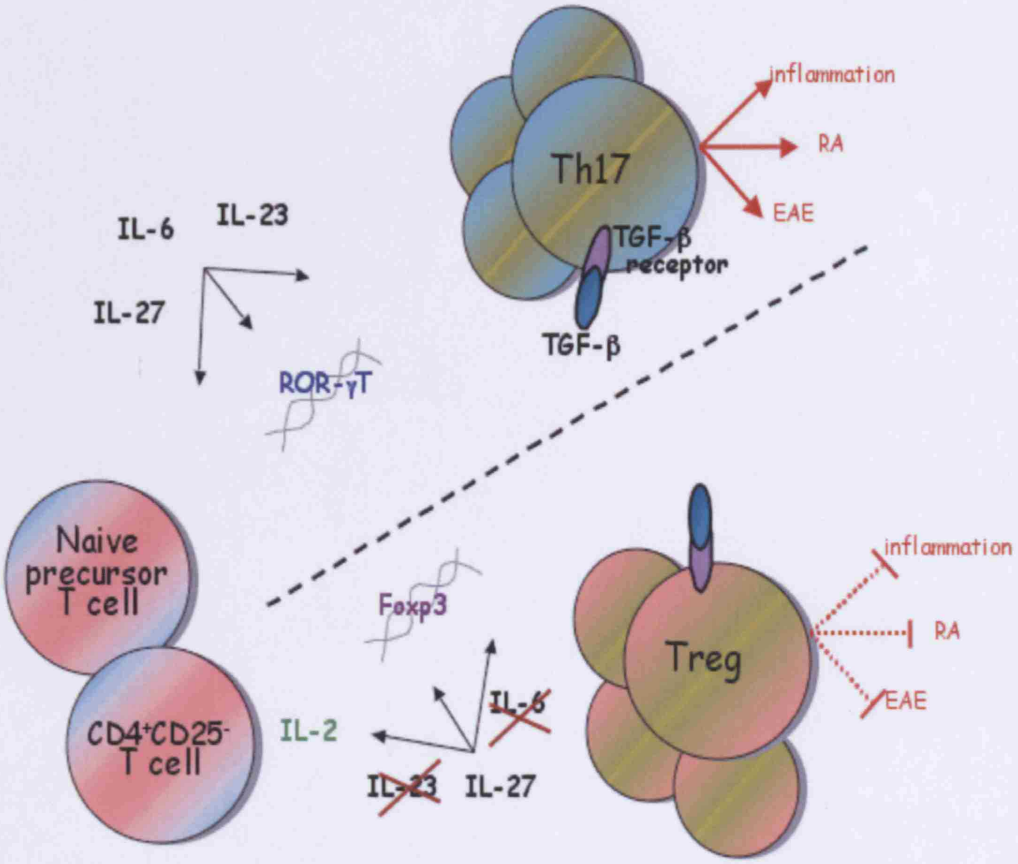


FIGURE 1.2.2.3 DISTINCT DEVELOPMENTAL PATHWAYS FOR TH17 AND REGULATORY T CELLS FROM COMMON T CELL PRECURSORS

IL-17-producing Th17 cells and regulatory T cells develop from common T cell precursors, both of which are dependent on TGF-β. Th17 cells develop in the presence of TGF-β and a "pro-inflammatory" environment, which includes, IL-6, IL-23 and IL-27. Development of Th17 cells is also dependent on expression of the transcription factor, ROR-γT. By contrast, Tregs develop in the presence of TGF-β, IL-2 and expression of Foxp3, but in the absence of a pro-inflammatory environment.

1.2.2.2 IL-10 IN REGULATORY T CELL DEVELOPMENT AND FUNCTION

IL-10 SIGNALLING PATHWAY

IL-10 was first described as cytokine synthesis inhibitory factor (CSIF), originally identified in mouse Th2 cells that inhibited the action and cytokine production of Th1 cells (160). In addition to inhibiting Th1 responses, IL-10 exerts its effects on macrophages and dendritic cells, inhibiting the release of specific factors produced by these cells, including IL-1, TNF-α, IL-6, GM-CSF and IL-12, as well as the expression of co-stimulatory molecules, CD80/CD86 and MHC class II molecules. Additionally, IL-10 blocks activation of the NF-κB pathway in macrophages *in vitro* (161), and stimulated human peripheral blood $CD4^+$ T cells (162). Consequently, IL-10 inhibits the activation and maturation of monocytes and T cells. IL-10 does, however, activate the NF-κB and AP-1 pathways in $CD8^+$ T cells, thereby promoting growth and differentiation of these cells (163).

The IL-10 receptor (IL-10R) is composed of two subunits, and both are members of the interferon receptor family. Each receptor is made up of a high-affinity, ligand-binding IL-10Rα chain, and an accessory IL-10Rβ chain, which has little effect on IL-10's binding affinity to the receptor. Expression of the two subunits differ: whilst the IL-10Rβ chain is constitutively expressed on most cells, the IL-10Rα chain is up-regulated on a range of cells, such as haemopoietic cells and T cells, following activation.

Upon ligand binding, IL-10R phosphorylates and activates the Jak family of tyrosine kinases -Jak1, which associates the IL-10Rα chain, and Tyk2, which associates with the IL-10Rβ chain, resulting in the phosphorylation and activation of STAT1α and STAT3 in T cells and STAT5 in monocytes (164).

IL-10 IN THE DEVELOPMENT AND FUNCTION OF PERIPHERAL REGULATORY T CELLS

The peripheral generation of another subset of Tregs is induced by IL-10. The discovery of these Tr1 cells came from *in vitro* experiments using human and mouse $CD4^+$ T cells. Following chronic TCR activation in the presence of IL-10, $CD4^+$ T cells differentiate into Tr1 cells that are characterised by their high levels of IL-10 production (as well as TGF-β and IL-5), together with low levels of IL-2 and no IL-4 production. Furthermore, these Tr1 cells are functionally suppressive, inhibiting antigen-specific naïve T cell responses *in vitro*, and are hypoproliferative in response to re-stimulation and are therefore anergic by definition (120). These initial experiments also demonstrated that Tr1 cells are functionally suppressive *in vivo*. Transfer of pathogenic $CD4^+CD45RB^{hi}$ T cells into SCID mice results in inflammatory bowel disease (IBD) (165). However, if these cells are co-transferred with Tr1 cells (even at very low numbers), IBD is prevented (120).

The phenotype of Tr1 cells differs from conventional, thymically derived Tregs, as they do not constitutively express the transcription factor Foxp3, both *in vitro* and *in vivo,* but Foxp3 is up-regulated following activation (166, 167). In contrast, Tr1 cells do express on their surface, like conventional Tregs, CD25, as well as the IL-15Rα chain. Expression of both receptor chains has been shown to be important in the expansion of Tr1 cells: in the presence of both IL-2 and IL-15, Tr1 cells are capable of expanding, and retaining their suppressive function, even in the absence of stimulation via the TCR, suggesting that IL-2 and IL-15 are important growth factors for Tr1 cells (168). Activation of Tr1 cells through the TCR augments expression of the normal activation markers, including HLA-DR, CTLA-4, CD40L and CD69, as well as expression of Th1- and Th2-associated chemokine receptors, CCR5, CXCR3, CCR3, CCR4 and CCR8 (168, 169).

Although DCs are important in the differentiation of Tr1 cells (see section 1.3), *in vitro* differentiation and expansion of Tr1 cells can occur in the absence of DC. For example, as described above, repeated stimulation in the presence of high doses of IL-10 induce Tr1 cells. Moreover, naïve T cells stimulated in the presence of OVA peptide in combination with the immunosuppressive drugs, dexamethasone and vitamin D3, results in the induction of IL-10 secreting regulatory T cells, that are capable of inhibiting EAE, but do not produce IFN-γ or IL-5 (170). $CD8^+$ Tr1 cells have also be described, that are anergic and functionally suppressive, and are induced via plasmacytoid DCs through CD40L-mediated co-stimulation (171).

In all conditions of Tr1 induction, however, IL-10 is secreted at high levels, and therefore is a signature cytokine of this subset of regulatory T cells. Thus, the cytokine profile of Tr1 cells varies, and is dependent on the conditions under which they are induced. *In vivo* experiments have shown that IL-10 alone is insufficient to induce Tr1 cells, since the cytokine is incapable of preventing expansion of pathogenic T effector cells. However, co-administration of IL-10 and rapamycin into diabetic mice that have been transplanted with allogeneic islet cells induces Tr1 cells and prolongs graft survival. This is because rapamycin is able to block IL-2-dependent expansion of T effector cells, but not IL-10- and TCR-mediated expansion of Tr1 cells (172).

Additionally, it has been demonstrated that administration of a non-activating (non-FcR binding) humanised monoclonal antibody against CD3 - hOKT3γ1(Ala-Ala) in patients with type 1 diabetes mellitus, improves insulin production after one year of treatment in 75% of patients (173). Moreover, administration of this monoclonal antibody coincides with increase levels *in vivo* of IL-10-producing $CD4^+$ T cells that are also $CCR4^+$, suggesting that disease improvement by hOKT3γ1 is mediated by the induction of Tr1 cells (174).

Suppression via Tr1 cells is mediated by IL-10 and TGF-β, suppressing both naïve and memory T cells. Induction of suppressive function of antigen-specific Tr1 cells is dependent on only initial TCR stimulation. Following activation, Tr1 cells engage in bystander suppression by local production of IL-10 and TGF-β. The effects of IL-10 and TGF-β include down-regulation of co-stimulatory molecules on APCs and their production of pro-inflammatory cytokines, such as TNF-α, as well as inhibition of T cell cytokine production and proliferation, which is mediated by TGF-β.

The production of IL-10 also induce anergic $CD4^+$ T cells in humans (175), whereas IL-10 and TGF-β (but not either cytokine on their own) is required to induce anergic T cells in mice (176). These induced anergic T cells suppress T cells in an antigen-dependent, cell contact-dependent, cytokine-independent fashion, and mediate their suppression by competing with antigen-activated T cells for the surface of the APC, as well as IL-2. In mice, adoptive transfer of IL-10 and TGF-β-induced anergic T cells is capable of prolonging allogeneic skin graft survival *in vivo*, and therefore inhibits graft Vs host disease (GvHD) (176).

1.2.3 REGULATORY T CELLS AND NON AUTOIMMUNE DISEASES

1.2.3.1 REGULATORY T CELLS AND GRAFT- VERSUS-HOST DISEASE

Transplantation of bone marrow can be successful in leukaemia patients; although, more recently, transplantation of allogeneic haematopoietic stem cells (HSCs) is the treatment of choice for leukaemia, as well as other disorders including lymphoma, sickle-cell anaemia, and aplastic anaemia. However, one of the major complications of bone marrow or HSC transplantation is graft-versus-host disease (GvHD), in which the allogeneic bone marrow recognises the recipient tissue as foreign, or the presence of alloreactive T cells from HSC transplants. Consequently, alloreactive T cells expand in response to the host's APCs, which subsequently results in differentiation of cytokine-producing and cytotoxic effector cells that cause inflammatory disease and severe tissue destruction.

Although donor T cells are the responsible for the pathology in GvHD, increasing evidence suggests that donor-derived regulatory T cells may suppress GvHD, and early experiments suggested that Tregs could have a therapeutic role in maintaining transplantation tolerance. Studies looking at transplantation in animals have shown the absence of Tregs exacerbates GvHD: depletion of donor $CD4^+CD25^+$ Tregs from the donor T cell inoculum or depletion of the host's Tregs prior to transplantation augments GvHD. However, if donor-derived Tregs are activated *ex vivo* and infused at the time of transplantation, then GvHD is significantly inhibited (177), with $CD62L^{+/hi}$ Tregs potently inhibiting GvHD (178). This may indicate that donor-derived Tregs preferentially home to the LNs of the host, suppress alloreactive donor T effector cells, and inhibit GvHD-induced pathology.

Early studies looking at SCID patients that have been transplanted with HLA-mismatched allogeneic stem cells showed that these patients do not develop GvHD. Further analysis revealed that these patients have high levels of serum IL-10, and a large proportion of donor-derived T cells produce significant amounts of IL-10 (179), suggesting that Tr1 cells may influence the response following transplantation. The importance of IL-10 in maintaining transplantation tolerance is also seen in bone marrow transplantation experiments in mice. These experiments show that inhibition of acute GvHD depends on transplantation of freshly isolated donor-derived (but not host-derived) Tregs that secrete IL-10 (180).

Analyses of the occurrence of GvHD in cancer patients that have received allogeneic HSC transplants have shown a direct correlation between IL-10 production from donor-derived T cells and disease inhibition: low frequencies of IL-10-producing donor-derived T cells correlates strongly with GvHD, whilst the converse is true in the presence of high frequencies of donor-derived T cell IL-10 (181).

1.2.3.2 REGULATORY T CELLS AND ALLERGY

Interaction of environmental and genetic factors, following exposure to specific allergens can lead to the development of an allergic response that is initiated by Th2 cells. The cytokine profile of Th2 cells (IL-4, IL-5 and IL-13) is responsible for promoting effector functions that include allergen-specific IgE production, eosinophil activation and recruitment, and bronchial hyperreactivity. However, exposure of healthy individuals to allergens results in a different outcome. For example, T cells from healthy PBMCs fail to proliferate in response to allergen, suggesting that regulatory T cells may be suppressing the immune response in healthy individuals, but are defective or lacking in allergic patients. Studies have shown that Tregs are defective in allergic patients in terms of their inability to suppress Th2 proliferation and cytokines, but are still able to suppress Th1 responses (182, 183). Additionally, IPEX patients lack functional $Foxp3^+$ Tregs and suffer from a severe allergic phenotype (such as dermatitis and hyper IgE syndrome), providing further evidence for defective Tregs promote allergic responses.

Initial experiments have shown that decreased IL-10 levels are inversely correlated with increased allergic responses (184). In support of these original observations, studies looking at the role of Tregs in allergic and healthy individuals have shown that upon exposure to environmental allergens, such as birch pollen or house dust mite, in allergic individuals, results in the development of a Th2 response. By contrast, the dominant immune response following exposure to the same allergens in healthy individuals is the production of allergen-specific Tr1 cells.

Moreover, comparison of the relative proportions of T cell subsets (i.e. Th1, Th2 and Tr1) between allergic and healthy individuals, revealed that healthy individuals have a higher proportion of Tr1 after allergen exposure (185). Common therapies used to treat allergy include antihistamines or glucocorticoids, such as dexamethasone. $CD4^+$ T cells from glucocorticoid-resistant patients fail to produce IL-10 upon stimulation in the presence of dexamethasone (187).

However, recent studies have shown that this defect can be reversed in the presence of vitamin D3, thereby enhancing the responsiveness of $CD4^+$ T cells to dexamethasone and increasing IL-10 production, thereby inhibiting Th2 cytokine production (186, 187). Furthermore, these latter studies demonstrated that the *in vitro* culture of $CD4^+$ T cells with dexamethasone and vitamin D3 induces allergen-specific Tr1 cells that are functionally suppressive. This is similar to previous *in vitro* studies that have induced Tr1 cells in the presence of dexamethasone and vitamin D3, which are capable of suppressing EAE (170).

1.2.3.3 Regulatory T cells and cancer

The immune system prevents tumour growth via immunosurveillance, in which a immune response will naturally eliminate tumours that arise spontaneously. Evidence for immunosurveillance in controlling tumours comes from animal studies. For example mice that lack lymphocytes and IFN-γ have higher incidences of spontaneous and chemically induced tumours (188). In addition to immunosurveillance, tolerance mediates the immune response to tumours: in murine models of tolerance, there are increased numbers of high-affinity, tumour specific T cells, suggesting that tolerant states promotes tumour survival, rather than deletion.

Additonally, patients receiving long-term immunosuppressive therapy (for example, following transplantation) are at increase risk of developing cancer. Consequently, evidence suggests that Tregs interfere with anti-tumour immunity in two potential ways: firstly, by inhibiting the generation of immunity to tumour antigens in the periphery, such as the LNs; and secondly, by neutralising tumour infiltrating effector T cells, that are important in tumour regulation.

The importance of Tregs in tumour immunity have been supported by observations that increased levels of Tregs are present in the peripheral blood of cancer patients, including breast, pancreatic (189), ovarian, and lung cancers (190), and increased numbers of Tregs are often correlated with poor prognosis, such as in ovarian cancers (191). These observations suggest that tumours promote the migration of Tregs at the sites of the tumours themselves, rather than to peripheral sites such as the LNs. Therefore, the tumour microenvironment is important in controlling tumour immunity. For example, B cells from Non-Hodgkin lymphoma patients secrete the chemokine CCL22, which attracts Tregs to the tumour site. Analysis of Tregs from these patients also revealed that these cells express high levels of CCR4 on their surface, which is the receptor for CCL22 (192).

Analysis of Tregs at tumour sites have shown that the vast majority secrete IL-10 and/or TGF-β and these cytokine are responsible for suppressing anti-tumour responses (193). Moreover, tumours can directly induce the generation of Tr1 cells, as observed in Hodgkin lymphoma patients (194), and $CD8^+$ Tr1 cells can be induced by tolerogenic plasmacytoid DCs that infiltrate the tumour microenvironment in patients with ovarian cancer (195). Further evidence that Tregs are induced by the tumour microenvironment comes from studies that show that T effector cells that are present in tumour draining LNs also secrete TGF-β and IL-10, which in turn, promote the induction of more Tregs (196), in a process similar to infectious tolerance (130).

The above evidence suggests that Tregs are a potential therapeutic target for cancer therapy, where careful depletion and/or inactivation of Tregs may result in augmented induction of effector T cells, which promote tumour-specific immunity. Initial studies that suggested Tregs might be therapeutic targets had shown that elimination of suppressor T cells with cyclophosphamide (a drug that mediates DNA cross-linking, and is commonly used to human cancers) results in immune-mediated degeneration of advanced lymphoma (197).

Support for this finding comes from animal studies that have shown the enhanced immunostimulatory effects seen with low doses of cylophosphamide, is due to the elimination of proliferating Tregs, but not other $CD4^+$ T cell populations (198). Factors that regulate Treg function have also been investigated as potential targets for cancer therapy. For instance, in vivo administration of blocking antibodies to CTLA-4 inhibits Treg function and promotes cancer regression; however, a major complication is that blockade of CTLA-4 results in severe autoimmunity (199).

1.3 AUTOIMMUNITY

1.3.1 INDUCTION AND CLASSIFICATION OF AUTOIMMUNE DISEASES

Under normal conditions, the immune system is able to eliminate cells that are reactive to self-antigens, thereby maintaining self-tolerance. However, depending on specific factors, adaptive immune responses against self-antigens fail to eliminate self-reactive cells, and persistence of such responses can lead to autoimmune pathology. Therefore, autoimmunity occurs when tolerance to self-antigens is broken, and can result from both genetic and/or environmental factors.

Autoimmune diseases can affect a particular organ, and thus be classified as organ-specific; or affect many organs around the body, and therefore be classified as systemic/multi-organ. Examples of organ specific diseases include type 1 insulin-dependent diabetes mellitus (IDDM), in which immune reactions, due to anti-insulin antibodies, destroy the individual's insulin-producing (islet) pancreatic cells. Other examples of organ-specific diseases include Graves' disease, which is characterised by the production of antibodies to the thyroid-stimulating hormone (TSH) receptor in the thyroid gland.

Examples of systemic autoimmune disease include systemic lupus erythematosus (SLE) and is characterised by the presence of antibodies to antigens that are ubiquitous and abundant in every cell of the body, such as anti-chromatin antibodies and antibodies to proteins of the pre-mRNA splicing machinery, known as spliceosome complexes that are present within the cells. Another example of systemic autoimmune disease includes rheumatoid arthritis (RA) and is discussed below.

The effector mechanisms that contribute to pathology from specific autoimmune diseases can also be classified according to how they mediate their effects. For example, pathology that results from IgG or IgM responses to auto-antigens located on cell surfaces or extracellular matrix can lead to autoimmune diseases such as the skin blistering disease, pemphigus vulgaris. Other effector mechanisms include immune complexes, which are typical of systemic autoimmune diseases such as SLE and RA, and contain auto-antibodies to soluble auto-antigens that are deposited at specific anatomical regions, such as joints in RA. Finally, auto-reactive T cell responses can cause pathology, and are typical in SLE and RA pathology. Therefore, autoimmune pathology often results from more than one effector mechanism.

1.3.1.1 Genetic susceptibility to autoimmune disease

Evidence for the genetic susceptibility of autoimmune diseases came from studies looking at monozygotic and dizygotic twins. For example, IDDM shows a concordance of ~40% in monozygotic twins, and 5% in dizygotic twins, and in SLE, around 20% of monozygotic pairs are affected, whereas only 3-4% are affected in dizygotic twins. Such studies suggest that if disease concordance is higher in monozygotic twins, then genetic factors play a major role in the induction of autoimmune disease; however, if the likelihood of a specific autoimmune disease occurs at equal frequencies in monozygotic and dizygotic twins, then both genetic and environmental factors are attributed to the disease. In addition to twin studies, studies looking at occurrence specific autoimmune disease within families, compared to the occurrence of that disease within the general population have been carried to determine heritability.

Since T cell responses rely on antigen presentation via the MHC, susceptibility of specific diseases often correlates with differences on specific HLA alleles, particularly MHC class II. For example, individuals that are heterozygous for HLA-DR3 and DR4 are more susceptible to IDDM, whereas individuals who carry HLA-B27 (an MHC class I allele) are more susceptible to ankylosing spondylitis. The implication of such MHC associations would influence the T cell response, particularly T cells that respond to self-antigens.

However, carrying specific HLA alleles alone does not necessarily lead to autoimmune disease, and other genetic factors, such as defects in specific proteins, are also attributed. The autoimmune regulator (AIRE) gene regulates autoimmunity by promoting the thymic expression of peripheral tissue-specific antigens, and thereby maintaining tolerance by regulating self-reactive T cells. However, genetic polymorphisms in the *AIRE* gene result in multi-organ autoimmune disease, known as autoimmune polyendocrinopathy syndrome type 1.

A susceptibility risk for a specific HLA allele in a specific autoimmune disease can also be calculated by comparing the observed number of patients carrying the HLA allele with the number that would be expected, which is estimated according to the prevalence of the HLA allele in the general population. For example, individuals that carry HLA-DR3/4 have a four-fold greater chance for development of IDDM, than do individuals who lack the HLA allele; and individuals carrying HLA-B27 are 100 times more like to develop ankylosing spondylitis. Additionally, the sex of the individual can increase susceptibility, where females, for example, are three times more likely to develop RA and ten times more likely to develop SLE.

1.3.1.2 Environmental factors that influence autoimmune disease

In addition to genetic factors, environmental factors can contribute to autoimmunity. For example, microbial infections can initiate or even enhance susceptibility to autoimmunity, since they are able to induce strong inflammatory reactions, which can lead to an infiltration of self-reactive T cells into target organs. This often results from cytokine release from bystander cells that are present during microbial infections, which in turn can lead to the activation of autoreactive T cells. Molecular mimicry is another way microbial infections can lead to autoimmune disease, where the microbe shares one or more epitopes (cross-reaction) with self-antigens, or mimics the self-antigen, which is then presented to and recognised by T cell. Such cross-reacting T cells have been shown to occur in IDDM and multiple sclerosis (MS).

Other environmental factors, such as the lifestyle of the individual can affect the outcome of the autoimmune disease. For example, smoking exacerbates the onset of pulmonary haemorrhage in patients with Goodpasture's syndrome, an autoimmune disease in which auto-antibodies against basement membrane or type IV collagen are produced and cause extensive vasculitis. Cigarette smoke damages the alveolar capillaries, by inducing an inflammatory response in the lung. Consequently, the auto-antigen is exposed to the autoantibody, resulting in a localised autoimmune response within the lung.

The exposure of environmental factors, such as microbial can lead to the prevention of allergic diseases and asthma. Recent changes in modern environments and lifestyles have lead in global rise in the prevalence of allergic disease in children. The hygiene hypothesis, which suggests that naturally occurring microbial exposures at early life may prompt early immune maturation and prevent allergic diseases and asthma from developing. Therefore, the absence of infectious exposure at a critical point in immune system development leads to a greater risk for the later development of allergic disease.

1.3.2 RHEUMATOID ARTHRITIS

1.3.2.1 CLINICAL FEATURES OF RHEUMATOID ARTHRITIS

RA is a systemic, chronic inflammatory disease, that is more common in women than men and affects 1% of the population. Characteristically, RA affects the peripheral joints, typically of the hands, feet, wrists and knees, in a symmetric fashion, leading to cartilage destruction, bone erosion, and subsequent joint deformity. In healthy individuals, the lining of the joints – the synovial membrane, serves to lubricate and nourish the joints through synovial fluid, which is composed of macrophage- and fibroblast-like synoviocytes that are situated on a fatty connective tissue lining called the sub-intima.

Early disease is characterised by pain and swelling of the hands and small joints of the feet, with only a mild inflammatory infiltration. As the disease becomes established, joints of the knees, ankles and hips, as well as the cervical

spine and temporomandibular joints (the lower jaw) become tender and swollen, and large inflammatory nodular masses are present within the joints. Additionally, disease progression correlates with high serum levels of rheumatoid factor (RF), for which around 80% of RA patients are seropositive.

There are a number of extra-articular manifestations resulting in inflammatory lesions in many tissues, including the heart, lungs (pulmonary fibrosis), subcutaneous tissue (subcutaneous rheumatoid nodules, particularly on the forearm), skin (cutaneous vasculitis, particularly nail beds of the fingers), salivary gland and lacrimal glands (Sjrogren's syndrome).

1.3.2.2.1 Diagnosis and assessment of RA disease activity

Laboratory investigations

One or more laboratory/immunological tests can be used to assess rheumatoid arthritis. Such tests are used for assistance in diagnosis, and results of laboratory studies are supportive or confirmatory of the clinical presentation based on an accurate, complete history and physical examination (see next sub-section). Common laboratory tests used for RA diagnosis involve measurement of acute phase reactants that are significantly raised by inflammation, which include the erythrocyte sedimentation rate and C-reactive protein (the acute-phase response), as well as measuring circulating levels of auto-antibodies, including rheumatoid factor and anti-cyclic citrullinated peptides.

Erythrocyte sedimentation rate (ESR)

The ESR is the rate of fall of erythrocytes in a column of blood in 1 hour, and is influenced by the extent to which erythrocytes are aggregated. Therefore, the ESR is directly proportional to the amount of erythrocyte aggregation and the size of the aggregates. However, ESR can be affected by other factors such as immunoglobulins, which can enhance erythrocyte aggregation. The level of the ESR generally correlates with the severity of inflammation or tissue injury.

Consequently, it is a non-specific test, which can be influenced by other factors such as underlying infection. In chronic infections or inflammatory conditions such as rheumatoid arthritis, the ESR may also fluctuate with increased disease activity and remissions. Normal rates for males are between 1-11 mm/hour and for females between 3-25mm/hour, and is slightly higher in older people.

C-REACTIVE PROTEIN (CRP)

CRP is a non-glycosylated protein produced by human hepatocytes in response to infection, inflammation, or tissue damage, which binds to the plasma membrane of damaged, but not living cells. Unlike ESR, CRP is not affected by the presence of factors such as immunoglobulins and is a more accurate measure of inflammation.

Following the onset of an inflammatory stimulus, CRP levels begin to rise within a few hours and peak within 48 hours, and levels are not affected by the sex or age of the individual. CRP is present at very low levels in the healthy individuals. Levels less than 1 mg/dl are considered insignificant, levels from 1 to 5 mg/dl are considered moderately elevated, and levels greater than 5 mg/dl are markedly elevated (200).

RHEUMATOID FACTOR (RF)

The most commonly used tests for IgM rheumatoid factor have been the latex and bentonite agglutination tests and the Rose–Waaler or sheep cell agglutination tests. In the Rose–Waaler test, sheep erythrocytes are coated with rabbit IgG. Serial dilutions of human serum are added to aliquots of Ig-coated sheep cells that are visibly agglutinated by the presence of IgM RF, and the highest dilution of serum that causes agglutination is the "rheumatoid factor titre."

Two other very sensitive methods that can be used to detect IgM rheumatoid factor are the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA), and the RF level is quantified by determining the optical

density of the substrate solution. Most patients with RA have elevated RF titers within a year of onset of the disease, although approximately 10 to 20% remain RF negative.

CLINICAL ASSESSMENT OF DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS

There are two main composite measures to assess disease activity in RA The first are the American College of Rheumatology (ACR) response criteria, and are based on a core set of disease activity variables, which include swollen joint counts (SJC), tender joint counts (TJC), as well as pain, physician global assessment, and the acute phase response, which incorporates ESR and CRP. The ACR criteria are expressed as ACR20, 50 70 and 90 and correspond to 20%, 50%, 70% and 90% improvement of the respective variables mentioned above from the baseline, and the score is based on percentage improvement in both SJC and TJC, plus three out of the five remaining variables (201). These disease activity criteria are commonly used when assessing the therapeutic efficacy of specific drugs for RA in clinical trials. Due to the use of baseline measures, the ACR criteria are categorical in nature and conclusions made on improvement are based on judgement rather than actual disease activity (see table 1.3.2).

The other measure used to asses disease activity is the disease activity score (DAS)-28, which is based on 28 joint counts, ESR and global health assessment, and is a continuous measure of disease activity. Joint damage is measured by assessing joint space narrowing and bone erosions, which can be seen on radiographs. The Sharp score is the most frequently used method for assessment of joint space narrowing. Unlike ACR criteria, DAS28 is not dependent on baseline parameters to assess any differences in disease activity. Consequently, DAS28 is a closer measure of disease severity than the ACR criteria.

Criterion	Definition
1. Morning Stiffness	Morning stiffness in and around the joints, lasting 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid observed by a clinician. Possible areas are PIP*, MCP, wrist, elbow, knee, ankle and MTP joints
3. Arthritis of hand joints	At least 1 area swollen in a wrist, MCP or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2.) on both sides of the body
5. Rheumatoid nodules	Subcutaneous nodule, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a clinician
6. Serum rheumatoid factor (RF)	Demonstration of abnormal amounts of serum RF by any method for which the result has been positive in <5% of normal controls
7. Radiographic changes	Changes typical of RA on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localised in or most marked adjacent to involved joints (not inc. osteoarthritis changes)

TABLE 1.3.2 ACR REVISED CLASSIFICATION CRITERIA FOR RHEUMATOID ARTHRITIS

The American college of rheumatology (ACR) revised classification criteria is a guide for clinicians to diagnose a patient with rheumatoid arthritis. The patient must satisfy at least 4 out of the 7 criteria listed above, and criteria 1-4 must be present for at least 6 weeks. PIPs – proximal intraphalangeal joints; MCPs – metacarpophalangeal joints; MTP – metatarsophalangeal joints.

1.3.2.3 PATHOGENESIS OF RHEUMATOID ARTHRITIS

CELLULAR INTERACTIONS IN THE JOINT PATHOLOGY OF RHEUMATOID ARTHRITIS

The cellular composition of rheumatoid synovitis comprises resident fibroblast-like and macrophage-like cells, as well as endothelial cells. The inflammatory comprises T and B cells, macrophages and neutrophils that invade the synovial membrane. This results in proliferation of the synoviocytes, which, as disease progresses, becomes hyperplastic that invade and destroy the articular cartilage and underlying bone. Consequently, inflammation of the synovium, or synovitis follows (see below).

The organisation of the inflammatory infiltrate during synovitis differs amongst individuals and affects the outcome of the immune response. Therefore, synovitis can fall into three distinct groups, based on cellular organisation:

1. The least frequent type of synovitis (only a few percent of all patients with RA) is characterised by granuloma formation, and is similar to rheumatoid nodules that form at extra-articular sites.

2. The most common type of synovitis is a diffuse inflammatory infiltrate, in which T and B cells, as well as macrophages, a distributed randomly amongst the tissue-resident cells, thereby having a disorganised appearance.

3. In about 50% of patients with RA, synovitis is characterised by T and B cells, and follicular DCs organising themselves into specific follicular structures, or lymphoid follicles. Synovitis in half this patient group comprises lymphoid follicles with distinct germinal centre formation, with separate T and B cell zones, as well as B cell proliferation and maturation – a similar structure to secondary lymph nodes. Synovitis in the other half of the patient group comprises lymphoid follicles which lack germinal centres, and T and B cells form aggregates and is characterised by a lack of follicular DCs.

In addition to the organisation of the inflammatory infiltrate, resident cells of the synovium undergo different levels of organisation, especially endothelial cells, that are primarily involved (in this case) in the process of angiogenesis. For example, in patients with synovitis that consists of germinal centres, blood vessels that form around T and B cell aggregates are more prominent. By contrast, in patients with synovitis that displays a diffuse organisation, very few blood vessels develop, suggesting that angiogenesis is favoured in synovitis where organised lymphoid follicles are present.

B CELLS IN RHEUMATOID ARTHRITIS PATHOLOGY

Following initial exposure to an antigen or pathogen, some B cells become memory cells and others differentiate into plasma cells. When the same antigen is encountered again, the cells are primed and the response raised against the pathogen is usually rapid and effective. Evidence also suggests that B cells are crucial in the orchestration of inflammatory pathways and provide important links between innate and adaptive immunity. Like T cells and macrophages, B cells produce pro-inflammatory cytokines. Moreover, they regulate the trafficking of immune cells firstly by producing chemokines and secondly by responding to chemokines through chemokine receptors expressed on their surface. In addition to the ability of activating APCs, B cells are efficient APCs themselves. Furthermore, they are not only involved in T cell activation and expansion, but are also crucial in the organisation and development of lymphoid tissue architecture. Evidence shows that whenever the B cell system is impaired or depleted, lymphoid tissue lacks proper organisation, and normal functioning of the immune system is disrupted.

B cells contribute to RA pathology in four ways:

- Autoantibody production
- Pro-inflammatory cytokine production
- Chemokine and chemokine receptor regulation and interactions
- Ectopic lymphoid organogenesis (see above)

AUTO-ANTIBODY PRODUCTION

Auto-antibodies form and enlarge immune complexes (when they bind to their auto-antigen) leading to activation of B cells and follicular dendritic cells (FDCs) via Fc receptors and complement receptors 1 (CR1) and CR2 (also known as CD21 and CD35, respectively) expressed on their surfaces, resulting in immune system activation. In addition, B cells express a specific B cell receptor (BCR). All these receptor systems, similar to the toll-like receptors, combine the innate and adaptive immune system on the surface of B cells. B cells, as well as other cells, can process immune complexes for antigen presentation, thereby enhancing local inflammatory processes.

Examples of auto-antibodies include RF and are auto-antibodies (usually of the IgM isotype) against the Fc portion of IgG that represent one of the two objective classification criteria of RA, detectable in about 80% of RA patients: high serum levels of RF correlates with disease progression. RFs are also synthesized in healthy individuals, but differ from RA-derived RFs, by the affinity maturation of the autoantibody: in healthy individuals, RFs are secreted by $CD5^+$ B cells and have a low affinity for IgG. RFs derived from RA patients, however, have high affinity for IgG (202).

Another example of auto-antibodies in RA includes auto-antibodies to citrullinated peptides such as cyclic-citrullinated peptides (anti-CCP) (203). Citrulline is a post-translational modification of arginine. In the presence of high calcium concentration (which can occur when cells die due to apoptosis) the enzyme peptidyl arginine deiminase (PAD), which is present in the granulocytes and monocytes that are abundantly present in an inflamed synovium, converts the positively charged imino group of arginine to the uncharged carbonyl group of citrulline. This increases the peptide affinity for shared epitope (SE) positive HLA-DRB1 molecules (see below).

CHEMOKINE INTERACTIONS AND ECTOPIC LYMPHOID ORGANOGENESIS

Distinct expression patterns of chemokine receptors CXCR3, 4 and 5 (as well as CCR5 and CCR9 on T cells – see below) in the blood of RA patients have been identified as compared to controls, indicating that there are changes in lymphocyte trafficking under disease conditions. Analysis of peripheral blood B cells from RA patients have shown that they express significantly lower levels of CCR6, CXCR4, and CXCR5, compared to healthy controls and osteoarthritis (OA) patients. However, RA B cells express significantly higher levels of CXCR3 (204). A number of chemokines have been identified that contribute to the formation of ectopic lymphoid tissue. Chemokine ligands including B-cell derived lymphotoxin-β (LT-β) and FDC-derived CXCL13 (also known as B lymphocyte chemoattractant; BLC), as well as CXCL21 are all very important in the generation of germinal centers (205, 206). While CXCL13 is involved in the recruitment of B cells, CXCL21 is responsible for attracting T cells and have been shown to be at lower levels in patients with a more diffuse synovitis (206).

T CELLS AND THE ROLE CYTOKINES IN RHEUMATOID ARTHRITIS PATHOLOGY

EVIDENCE FROM MURINE STUDIES

T cells are recruited into the synovium via the expression of specific chemokines, and those that migrate into the joint are usually activated effector memory $CD4^+CD45RO^+$ T cells. Adoptive transfer of $CD4^+CD28^-$ T cells into human (RA synovium engrafted) tissue-SCID mice chimaeras results in these cells expressing CCR5, CCR7 and CXCR4 and preferentially homing to the RA synovium tissue. Moreover, IL-12 plus TCR stimulation redirects homing from the LNs to the synovial tissue via up-regulation of CCR5 expression (207). Initial experiments carried out in animal models of RA suggested that RA pathology is solely driven by Th1 responses. For example, studies looking at murine collagen-induced arthritis (CIA) have shown that Th1-derived cytokines are increased during RA pathology.

Murine CIA is an autoimmune disease that is induced via immunisation with cartilage derived from type II collagen, resulting in collagen-specific T cell responses. The resultant pathology is a destructive inflammatory response that affects the joints, which resembles RA. Original experiments analysing the cytokine profiles from these mice demonstrated that cultured draining LNs produced high levels of IFN-γ, which correlated with disease progression (6 days after immunisation). Moreover, the increase in IFN-γ coincided with a complete suppression of Th2-derived cytokines (208). Subsequent studies have shown that CIA mice treated with IL-12 and IL-18, two cytokines that drive Th1 responses, augments disease progression and severity compared to untreated CIA mice, and neutralisation of both these cytokines reduces disease (209).

In another animal model for RA, SKG mice spontaneously develop severe arthritis under normal conditions, but not under specific pathogen-free (SPF) conditions (i.e. a microbially clean environment). However, disease can develop under SPF conditions if self-reactive T cells are initiated to proliferate.

Recent evidence suggests that induction of arthritis in this strain of mice under SPF conditions is due to the differentiation of self-reactive T cells into Th17 cells (210). This suggests that differentiation of self-reactive T cells into Th17 cells, and hence disease progression, is dependent on the cytokine milieu, since SKG mice that are deficient in APC-derived IL-6 lack Th17 development and do not develop arthritis (210).

Further evidence for IL-17 in the pathology of RA comes from studies looking at CIA mice that lack Th1-associated genes including *ifng, ifngr* or *il-12(p35)*. In these mice, collagen-induced arthritis is accelerated, and not suppressed, as would be expected if pathology is driven by Th1 response (211, 212). By contrast, mice that are deficient in cytokines genes that promote/maintain Th17 cell differentiation, namely, *il-6* and *il-23(p19)*, are protected from collagen-induced arthritis (144, 213).

EVIDENCE FROM CLINICAL STUDIES IN RA PATIENTS

In addition to animal models, clinical studies have shown that RA patients who also suffer from allergic diseases, such as hay fever, have less severe disease, as determined by ESR, CRP and radiographic joint damage scores, compared to RA patients who do not suffer from allergic diseases. The attenuated disease activity in RA-allergic patients is attributed to the fact that Th2 responses dampen down Th1 responses, and therefore improves pathology in RA (214).

More recently, studies have suggested that Th1 responses are not as important in RA pathology, as originally thought. For example, analysis of the synovial membrane from patients with RA reveals that IFN-γ is present at very low levels, or even lacking, and is undetectable in the synovial fluid of the same patients (215).

Interactions between T cells and macrophages promote the release of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6. For example, IL-15, a growth factor for synovial T cells that is produced by macrophages,

DCs and B cells, promotes T cell-induced TNF-α and IL-6 production from macrophages in a cell contact-dependent manner (216). Additionally, synovial fibroblasts, as well as synovial T cells release TGF-β, and together with IL-6, an ideal cytokine environment is established for the development of Th17 cells. In addition to neutrophils differentiation, maturation and recruitment, IL-17 activates monocytes, resulting in chemokine and cytokine release.

IL-17 has also been shown to work in synergy with TNF-α and IL-1β at low concentrations, leading to synovial fibroblast activation and cytokine production (217), and all three pro-inflammatory cytokines, as well as IL-6, are directly involved in joint damage: the balance between bone formation and bone resorption in RA is skewed towards the latter, and is mediated by osteoclasts.

Osteoclasts are at the interface between synovial tissue and the articular bone. By promoting bone resorption, osteoclasts allow the infiltration of cells from the synovial membrane that subsequently leads to pannus formation (a layer of inflammatory granulation tissue comprising of inflammatory cells, blood vessels and fibrous cells), which spreads from the synovial membrane and ultimately into the joint. M-CSF and RANKL (receptor activator of NF-κB ligand - a member of the TNF superfamily) are both necessary for osteoclasts differentiation. TNF-α induces M-CSF production from synovial fluid cells. IL-1β, IL-6, TNF-α and IL-17 all up-regulate RANKL expression on synovial fibroblasts (218-220).

GENETIC FACTORS CONTRIBUTING TO RHEUMATOID ARTHRITIS: HLA- AND NON HLA-ASSOCIATED GENES

Indication of familial susceptibility of RA came from studies that demonstrated familial clustering of the disease. The prevalence of RA ranges between 2-15% in first-degree relatives (i.e. parents to offspring and siblings), compared to a prevalence rate of 1% within the general population. Concordance rates of RA in monozygotic twins range between 12-30%, and is halved in dizygotic twins, indicating that although genetic factors have major implications in the development of RA, environmental factors also play a role (221, 222).

Original studies demonstrated an association between HLA genes and the prevalence of developing RA. In these studies it was shown that HLA molecules within the HLA-DR1 gene locus *HLA-DR4* (also referred to as HLA-DRB1*0401), is tightly linked with RA progression, with 70% of RA patients carrying the HLA-DR4 allele, compared to 28% of normal controls (223). The association of HLA-DR4 in the genetic susceptibility of RA is also confirmed by familial studies, which have shown that the frequency of HLA-DR4 is higher in patients with familial RA (~70%), compared to patients with non-familial RA (~54%) (224).

Subsequent studies have identified several HLA-DRB1 alleles that are associated with RA, which include alleles encoded by HLA-DR1 (*0101, *0102), as well as alleles encoded by HLA-DR4 (*0401,*0404 and *0405), HLA-DR5 (*1102), HLA-DR6 (*1301), and HLA-DR10 (*1001). All these genes increase the susceptibility of RA by binding to the same peptides and triggering autoimmune disease, thereby forming the shared epitope hypothesis. This hypothesis explains why the class II region of the MHC is associated with RA, since all the products of the RA- associated alleles share a five amino acid sequence – $Q/K/R^{70}RRAA^{74}$ situated in the third hypervariable region of HLA-DRB1 (225).

Although evidence suggests that HLA-DRB1 is the main genetic factor in RA, the HLA locus only contributes to around 30% of total familial risk, suggesting that other non-HLA associated genetic factors are involved in RA. Non HLA-associated genes include TNF-α receptor 2 (*TNFR2*) gene, which is located on the short arm of chromosome 1(1p36). A single nucleotide polymorphism (SNP) found in *TNFR2*, originally identified in Japanese patients with SLE, results in the substitution of an arginine residue for a methionine residue at position 196, exon 6 (196R) that consequently increases the efficiency of intracellular transduction pathways when TNF-α binds to TNFR2 (226). This 196R substitution has also be identified in a significant number of British and French Caucasians patients who have familial RA, suggesting that this TNFR2 SNP may increase the risk factor of familial RA (227, 228).

Another non HLA-associated gene that has been attributed to increasing the genetic susceptibility of RA is the *PADI4* gene. This gene is also located at the same position as *TNFR2* 1p36, a locus that contains the peptidyl arginine deiminases (PADs), which catalyse citrullination of arginine residues. The resultant citrullinated epitopes cause the development of anti-CCP auto-antibodies, as well as other auto-antibodies specific for RA, but not RF. A study carried out in Japan has shown that eight SNPs in the *PADI4* gene are associated with familial RA, particularly where thymine is substituted by cytosine (padi4 *94 T/C), suggesting that the PADI4 haplotype is associated with susceptibility to RA due to increases in production of citrullinated peptides acting as auto-antigens, augmenting the risk of developing the disease (229). However, a similar study carried out in the UK has shown that the frequency of the *PADI4* haplotype was similar in both RA patients and controls (230), suggesting that the association between PADI4 and RA may differ between different populations.

The gene encoding the intracellular protein tyrosine phosphatase nonreceptor type 22 (PTPN22), has recently been implicated in susceptibility to RA. A missense SNP in the *PTPN22* gene, where cytosine is replaced by thymine results in a minor T allele in which arginine is substituted by tryptophan (R630W). This minor T allele has a significant association with Caucasian RA patients (28%), compared to healthy Caucasian controls (17%) (231). This substitution disrupts the P1 proline-rich motif that is important for interaction with the protein kinase Csk, which may alter normal function of PTPN22 as a negative regulator of T cell activation.

Other genes have recently been associated with RA susceptibility. For example, two SNPs mapping to the *CTLA-4* gene were found to be weakly associated with RA (and also type 1 diabetes) susceptibility. Of particular interest strong associations between RA susceptibility and SNPs mapping close to the *CD25* (as well as the IL2Rβ chain) gene were identified (232).

1.3.2.4 NON ANTI-TNF THERAPIES USED TO TREAT RHEUMATOID ARTHRITIS

The use and type of disease modifying anti-rheumatic drugs (DMARDs) to treat RA have changed considerably over the last few decades. For example, RA was treated using non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal drugs, such as glucocorticoid. In the past, DMARDs were started late into the disease for the treatment of RA. However, the current practice is to start therapy early on in disease in order to prevent joint damage. Conventional DMARDs include gold therapy, cyclosporine A, sulphasalazine, hydroxychloroquine and leflunomide. The most commonly used DMARD is methotrexate (MTX) and works by inhibiting T cell and macrophage function and decreasing IL-1 and IL-2 production (see table 1.3.2.4). In clinical trials, measuring the effectiveness of a drug involves assessing disease activity in RA (usually via the ACR criteria) and also assesses the impact on functional disability and quality of life.

As understanding of RA pathogenesis improved, the type of therapy used for RA changed, and various factors in the immune system that cause RA pathology, such as cytokines, became drug targets. Inflammatory cytokines usually exert their effects after binding to the cell surface receptor, and there are a number of ways to inhibit their effects: Soluble receptors or monoclonal antibodies bind to the cytokine and compete for binding with the cell surface receptor. Alternatively, receptor antagonists or monoclonal antibodies bind to the cell surface receptor and prevent the cytokine from binding.

One of the earliest approved biological response modifiers is anakinra, an IL-1 receptor antagonist. It is administered as a daily subcutaneous injection and has efficacy in treating RA when used alone or in combination with MTX. However, the use of anakinra is not currentlty as common due to the advent of anti-TNF-α therapies (see next section).

Newer biological modifiers include rituximab, a B cell depletion agent that is a chimeric mouse/human monoclonal antibody directed against CD20 antigen, which is expressed on the surface of mature- and pre- B cells, but not plasma cells (233). Rituximab is used in RA patients who have failed conventional DMARD therapy, as well as anti-TNF-α therapy. Clinical trials have shown that disease activity is improved greatly if rituximab is given in combination with MTX (10-25mg/wk) (234).

Abatacept is another recently identified biological modifier. It is a fusion protein linking the extracellular domain of human CTLA-4 to the Fc portion of human IgG1. It works by competing for binding between CD28 on the T cell and CD80/86 on the APC. Abatacept is usually given to patients who have failed one or more anti-TNF-α therapies, and have inadequate responses to MTX (235)

Tocilizumab is a humanised anti-IL-6 receptor monoclonal antibody. The IL-6 receptor complex is made up of two types of membrane proteins: IL-6 receptor α chain (CD126), which forms the non-signalling, IL-6-binding portion of the

receptor complex. In addition, two signal-transducing chains, the IL-6 β chain, gp130 (CD130), which dimerise, and form a trimolecular complex with the α chain. Tocilizumab targets the IL-6 α chain, which prevents IL-6 from binding to its receptor, and is efficacious when given at 4-10mg/kg as an infusion (236).

Drug name	Main mechanism of action	Effect	Common side-effects
Gold	Inhibits transcription factor AP1 binding.	Decreased MHC II expression on monocytes; inhibition of synovial cell proliferation; reduced IL-1 and lymphocyte proliferation	Hypersensitivity reactions, proteinuria
Cyclosporine A	Binds to intracellular phosphatase, calcineurin	Decreased IL-2, IFN-γ and NK function	Renal and hepatic impairment, GI upset
Sulfasalazine	Scavenges pro-inflammatory reactive oxygen species	Reduced numbers of activated lymphocytes	Rash, GI upset, hepatotoxicity
Hydroxychloroquine	Interferes with protease function and release; inhibits IL-1	Altered lysosome function, monocyte function and Ag processing. Decreased IL-1	GI upset, rash, headace and retinopathy
Leflunomide	Inhibits cell cycle of activated cells	Creased lymphocyte immune function. Inhibition of Ag processing	Hypertension, hepatic impairment, GI upset, headache, fatigue
Methotrexate	Increases adenosine levels; down-regulates purine nucleotides	Reduced leukocyte trafficking, inhibition of T cell and macrophage function, decreased IL-1 and IL-2	Heaptic impairment, GI upset, headache and fatigue

TABLE 1.3.2.4 TRADITIONAL NON-ANTI-TNF-α THERAPIES FOR RHEUMATOID ARTHRITIS

1.3.2.5 ANTI TNF-α THERAPIES USED IN RHEUMATOID ARTHRITIS

Initial *in vitro* experiments using cultures of dissociated rheumatoid synovial membranes, indicated that TNF-α would be an extremely beneficial target in RA therapy. In these experiments, blockade of TNF-α resulted in significant inhibition in IL-1 (237). Subsequent experiments also demonstrated a significant down-regulation of synovial-derived GM-CSF, IL-6 and IL-8 (238). These experiments therefore suggested that anti-TNF-α antibodies does not simply block TNF-α alone, but also has effects on other pro-inflammatory cytokines implicated in RA pathology.

Infliximab, adalimumab and etanercept are three anti-TNF-α therapies that are commonly used to treat RA in patients that have failed conventional, non-biological DMARDs. All three therapies can be used alone, but clinical trials (ASPIRE for infliximab (239); PREMIER for adalimumab (240); and TEMPO for etanercept (241)), have demonstrated that combination therapy with MTX significantly enhances the efficacy of each of the anti-TNF-α therapies, with ACR 20, 50 and 70 responses improving from 25% (infliximab alone) to 60%, 5% (adalimumab alone) to 20%, and 10% (etanercept alone) to 40%.

Adverse effects of anti-TNF-α therapies are rare; however, the commonest adverse events were related to the parenteral administration of the drugs, with one-third of etanercept-treated patients reporting injection site reactions, and 10% of infliximab-treated patients developing infusion reactions. Other common adverse effects with anti-TNF-α therapy include bacterial sepsis, and high risk of reactivation of latent tuberculosis (TB) early in the course of treatment; however, this is not observed in etanercept patients (see section 1.3.3.5). Therefore, patients are usually screened for TB prior to initiation of therapy, and any latent TB is treated at least one month before starting anti-TNF-α. Risk of drug-induced lupus may also be increased, since SLE is associated with low levels of TNF-α. Due to reduced levels of TNF-α, incidence of malignancies may also be increased in anti-TNF-α patients (since

TNF-α normally functions as immune surveillance for malignancies). However, so far, no increased incidences of malignancies have been observed.

ETANERCEPT (ENBREL ™)

Etanercept is a recombinant fully human protein, and its structure consists of two p75 TNF receptors attached to an IgG1 Fc region of a human immunoglobulin molecule. This construct is administered subcutaneously (s.c.) twice weekly (mean half-life, approximately 3 days) and is capable of significantly lowering levels of circulating TNF.

Etanercept has been shown to be significantly more effective in RA patients when compared with a placebo (242); however, one of the major disadvantages of this therapy is the need for repeated injection, as well as the high costs compared to conventional therapies. Randomised controlled studies have demonstrated the efficacy of etanercept. A double-blind (i.e. researcher/clinician and patient do not know which therapy is given) randomised trial of 234 patients with active RA given placebo or 25 mg twice-weekly etanercept s.c. injections. In this trial, combination therapy with MTX was not used. The ACR responses in those on etanercept were similar to those seen with infliximab on the 3 mg/kg 8 weekly regimen, with much improved disease activity compared to those that received placebo (242).

In the TEMPO (Trial of Etanercept and MTX with Radiographic Patient Outcomes) study, 686 RA patients with active disease who had not received MTX therapy were randomised to receive either etanercept (25 mg twice weekly, s.c.), or MTX (20mg weekly, orally), or a combination of both for 52 weeks. The study showed that patients receiving the MTX/etanercept in combination are more likely to improve than patients receiving etanercept or MTX alone (241). In addition to RA, etanercept has been approved to treat skin psoriasis and juvenile idiopathic arthritis (JIA).

INFLIXIMAB (REMICADE ™) AND ADALIMUMAB (HUMIRA ™)

Infliximab was the first anti-TNF-α therapy to be introduced, and both infliximab and adalimumab are monoclonal antibodies directed against TNF-α, especially membrane-bound TNF-α. In addition to treating RA, infliximab is effective in skin psoriasis and Crohn's disease. Infliximab is a chimeric antibody: The hypervariable region of the antibody is murine in origin, while the remainder of the immunoglobulin consists of a human IgG1Fc heavy chain and partial κ light chain.

Due to the chimeric nature of the antibody, it should have less potential to generate an immune reaction than a fully murine molecule but more potential than a fully humanized construct. Repeated intravenous administration has been associated with the development of clinically relevant human anti-chimeric antibodies (HACA) to the mouse component of monoclonal antibody in about 40% of RA and 61% of Crohn's patients. Consequently, infliximab is given simultaneously with MTX to abrograte the response of these antibodies, which could limit the long-term effectiveness of the therapy. Infliximab is administered intravenously, initially at weeks 0, 2 and 6, then every 8 weeks at 3mg/kg in combination with MTX. In contrast, adalimumab is a fully humanised monoclonal antibody and is administered by subcutaneous injection, given at 40mg every two weeks.

In addition to direct suppression of pro-inflammatory cytokines, infliximab directly affects leukocyte trafficking into the joints. Analysis of endothelial adhesion within the joint, following infliximab therapy revealed that serum levels of ICAM-1, E-selectin and VCAM-1 are all significantly reduced, which abrogates endothelial adhesion and activation, limiting leukocyte migration into the joint (243). Adding further support to this finding, immunohistological analysis of synovial membrane biopsises from RA patients before and after infliximab therapy, showed that expression of the above cell adhesion molecules are also decreased together with decreased cellularity in the synovium following therapy (244).

Investigation into the role of infliximab therapy in Crohn's disease has shown that infliximab promotes apoptosis of monocytes, which may account for the amelioration of disease following infliximab therapy (245, 246). However, in the case of RA, studies have been conflicting, where some have demonstrated that infliximab does not promote apoptosis of monocytes (or lymphocytes) (247), whereas others have shown that infliximab does induce apoptosis of monocytes, but not lymphocytes (248).

Various studies have compared chemokine and chemokine receptor expression on peripheral blood T cells between infliximab-treated and non-treated RA patients. These studies have shown chemokine receptor expression such as CCR3, CCR5 and CXCR3 are all up-regulated on peripheral blood $CD4^+$ and $CD8^+$ T cells, as well as on monocytes in RA patients following infliximab treatment (249, 250). These studies suggest that infliximab improves pathology by not only modifying cell adhesion expression in the joint, but also promotes accumulation of lymphocytes in the periphery and prevents their infiltration into the joint.

1.3.2.6 Mechanisms of action of anti-TNF-α therapies

Induced apoptosis

Evidence from patients with Crohn's disease suggests that infliximab induces apoptosis in activated monocytes and T-cells (245, 246). Assays of intestinal biopsies from Crohn's disease patients taken 24 hours post infliximab infusion found an increase in apoptotic CD3+ cells (245). Furthermore, it was shown that infliximab induced apoptosis in activated but not resting T-cells *in vitro*. These results suggest that infliximab may exert its sustained therapeutic effects in IBD by causing apoptosis of T-lymphocytes since uncontrolled T-cell activation plays a central role in IBD pathogenesis. Apoptosis has also been observed in circulating monocytes from Crohn's disease patients following infliximab infusion (246). Interestingly, the apoptotic effect of infliximab on T cells is not seen in the presence of etanercept (251).

Recently, it has been demonstrated that both etanercept and infliximab can induce apoptosis in monocytes and macrophages, but not T cells, from patients with RA, and this effect was more prominent in cells from the synovial fluid than from peripheral blood; no such effect was observed in lymphocytes (248).

ANTIBODY-MEDIATED CELL LYSIS

Cells coated with antibody isotypes that fix complement and bind Fc receptors (such as human IgG1) can activate complement- and antibody-dependent cell lysis. Infliximab induces complement- and antibody-dependent cell-mediated cell lysis in a murine myeloma cell line expressing membrane-associated TNF (252). Since macrophages and monocytes are among the cells that express membrane-associated TNF, the monocytopenia observed in patients following treatment with infliximab, suggest that this is probably due to infliximab causing the targeted killing of cells expressing membrane-associated TNF (253). Since monocytes are an essential component of the protective granuloma formation in TB, this pathway of cell lysis may explain the increased incidence of TB in infliximab-treated patients. Adalimumab may have similar activity because its effector portion is identical to that of infliximab (IgG1).

Etanercept contains the Fc portion of IgG1, but has been shown not to fix complement (254). Furthermore, because etanercept binds only single molecules of TNF, it is unlikely to form aggregates that can activate complement-dependent cytolysis and antibody-dependent cell-mediated cytotoxicity. Taken together these results suggest why there is no increase incidence of TB in etanercept-treated patients.

AIMS OF PHD

The initial objective of this PhD was to explore the Treg defect in RA. Previous work done in our lab has shown that Tregs from patients with active disease are defective in terms of their inability to suppress T-effector-derived pro-inflammatory cytokines (TNF-α and IFN-γ), a characteristic function of Tregs. In addition, it had been shown that patients treated with the TNF-α-neutralising drug, infliximab, have a restored Treg function, where the suppressor cells are able to suppress pro-inflammatory cytokine production.

This work prompted the following questions, which form the aims of the PhD

1. Do Tregs from active RA have an altered phenotype compared to healthy controls and patients treated with infliximab?
2. Since there is a restoration of function in Tregs from patients treated with infliximab, is TNF-α directly responsible for any phenotypic changes in RA Tregs?
3. Does the neutralisation of TNF-α (by infliximab) directly restore function in active RA Tregs, or is the drug able to induce a new population of Tregs that are not defective?
4. Does the defect solely lie within the Treg population, or are T effectors from active RA patients also defective – are they unresponsive to Treg suppression?

CHAPTER 2

MATERIALS & METHODS

All techniques carried out under sterile conditions in class II safety cabinets, unless otherwise stated. Full recipes of all buffers used can be seen in Appendix II

2.1 ACQUISITION AND STORAGE OF PATIENT AND CONTROL SAMPLES

2.1.1 ETHICAL APPROVAL AND PATIENT SELECTION

2.1.1.1 ETHICAL APPROVAL

Ethics for this study were approved by the Royal Free and University College London Medical School. Ethics approval number 02/0240. All subjects used, including healthy volunteers, were required to sign and date consent forms, prior to taking blood. See appendix I for patient consent form.

2.1.1.2 SELECTION OF PATIENTS

Patients used in this study were recruited according to the revised classification criteria of the American College of Rheumatology for RA (see section 1.5.2.1). Active RA patients were recruited according to their disease activity scores (DAS28), C-reactive protein (CRP) levels (greater than 5.0mg/dl), and that they had received no prior anti-TNF-α therapy. Infliximab patients received infusions at weeks 0, 2, 6, and at 8 -week intervals thereafter, given at a dose of 3mg/kg, in conjunction with methotrexate, given orally, at a dose of 7.5-15mg/kg/week. In addition to Infliximab patients, patients receiving Etanercept (25mg/kg, twice weekly, subcutaneous injection) were recruited for comparative studies. See Appendix I for sample patient information.

2.1.1.3 HEALTHY CONTROLS

Healthy controls were recruited on the basis that they did not have any history of autoimmune disease, and did not suffer from any anaemic conditions. Age- and sex-matched controls were used where possible.

2.1.2 STORAGE OF SAMPLES

Following isolation of PBMCs, cells were frozen down in freezing medium, at 1X 10^7 cells per ml. Freezing medium comprises of 10% Dimethylsulfoxide (DMSO, Sigma-Aldrich) in heat-inactivated foetal calf serum (FCS, Sera Laboratories International, Ltd). Cells were stored at –80°C for 72 hours and then transferred to liquid nitrogen.

When cells were needed for experiments, samples were quickly defrosted, by incubating vials in 37°C water baths. Once thawed, cells were re-suspended in warm sterile FCS, to minimise damage to cells by DMSO, and then topped up with warm RPMI-1640.

All plasma and supernatants were stored at –80°C.

2.2 CELL ISOLATION

2.2.1 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

50mls of peripheral blood were collected in heparinised tubes. Prior to PBMC isolation, blood was spun down at 420 X g at room temperature, in order to collect plasma. The remaining blood was made back up to 50mls with RPMI-1640 (Sigma-Alrdich). PBMCs were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (GE Healthcare), at a 1:2 Ficoll: blood ratio. Layered blood was spun at 800 X g at room temperature for 30 minutes. PBMC were carefully collected at the interface, and washed in 20mls RPMI-1640 (at 420 X g, 10 minutes) to remove any excess Ficoll. PBMC were re-suspended in 20mls of RPMI-1640. At this point cells were counted in order to determine cell number. The process of cell counting is as follows:

10µls of cells were mixed with 10µls of trypan blue, with 10µls of the mix loaded onto the haemocytometer.

All cells were counted within the 5 X 5 middle grid, any cells that lay on the inner line of the triple line were included, but not on the middle or outer lines.

Total cell number per ml was determined using the following equation:

Counted cell number X 2 (dilution factor) X20 (final volume) X 1 X 10^4

2.2.2 ISOLATION OF REGULATORY T CELLS USING

MAGNETICALLY-LABELLED BEADS

All reagents and equipment used for this method for Treg isolation was purchased from Miltenyi Biotec. All antibody incubations and washings were carried out at 4°C. The isolation of Tregs from PBMC using magnetically labelled beads requires two processes: Firstly, the depletion of all non-$CD4^+$ T cells, with a second process of a positive selection of $CD4^+CD25^+$ T cells.

DEPLETION OF ALL NON-$CD4^+$ T CELLS

First, cells were stained with a Biotin-conjugated antibody cocktail*, at a concentration of 10µls/10^7 cells, and incubated for 10 minutes, followed by the addition of anti- Biotin microbeads (20µls/10^7 cells) and a further incubation period of 15 minutes. Cells were then washed in 2mls of cold MACS® buffer at 300 x g for 10 minutes. Once the supernatant was completely removed, cells were re-suspended in 500µls of buffer, ready for magnetic separation. Prior to the depletion process, LD columns were primed by the addition of 2mls of buffer (to ensure that azide, which is present in the columns, was completely removed before the addition of cells). Cells were then added to the columns, slowly so as to minimise any bubbles that may block the flow of cells. The column was washed with 3 X 2mls of buffer once the cells had completely passed through. Cells that had now passed through the column were unlabelled $CD4^+$ T cells.

POSITIVE SELECTION OF $CD4^+CD25^+$ T CELLS

Unlabelled CD4+ T cells were washed as above, the supernatant completely removed, and re-suspended in 90mls of cold buffer. Cells were stained with a fixed volume of CD25 microbeads (10µls), mixed thoroughly, and incubated for 15 minutes. Cells were then washed in 2mls of buffer, supernatant completely removed, and re-suspended in 500µls of buffer. As with the LD columns, MS columns were also primed with buffer (500µls) prior to use. Cells were passed through the column, and washed (3 X 1ml) with buffer once

cells had completely passed through. In this process, cells that passed through were CD4+CD25- T cells, with the CD4+CD25+ fraction remaining within the column.

These cells were removed with the use of the supplied plunger. In order to increase the purity of the CD4+CD25+ fraction, these cells were passed through a freshly primed MS column, with steps repeated as before.

Purity of MACS®-separated regulatory T cells was characteristically around 95% pure. Representative purity FACS plots of both the T effector and regulatory T cell populations can be seen in figure 2.2.2

* The CD4+ T cell biotin antibody cocktail consists of biotin-conjugated anti-human antibodies against:

CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/∂, and glycophorin A

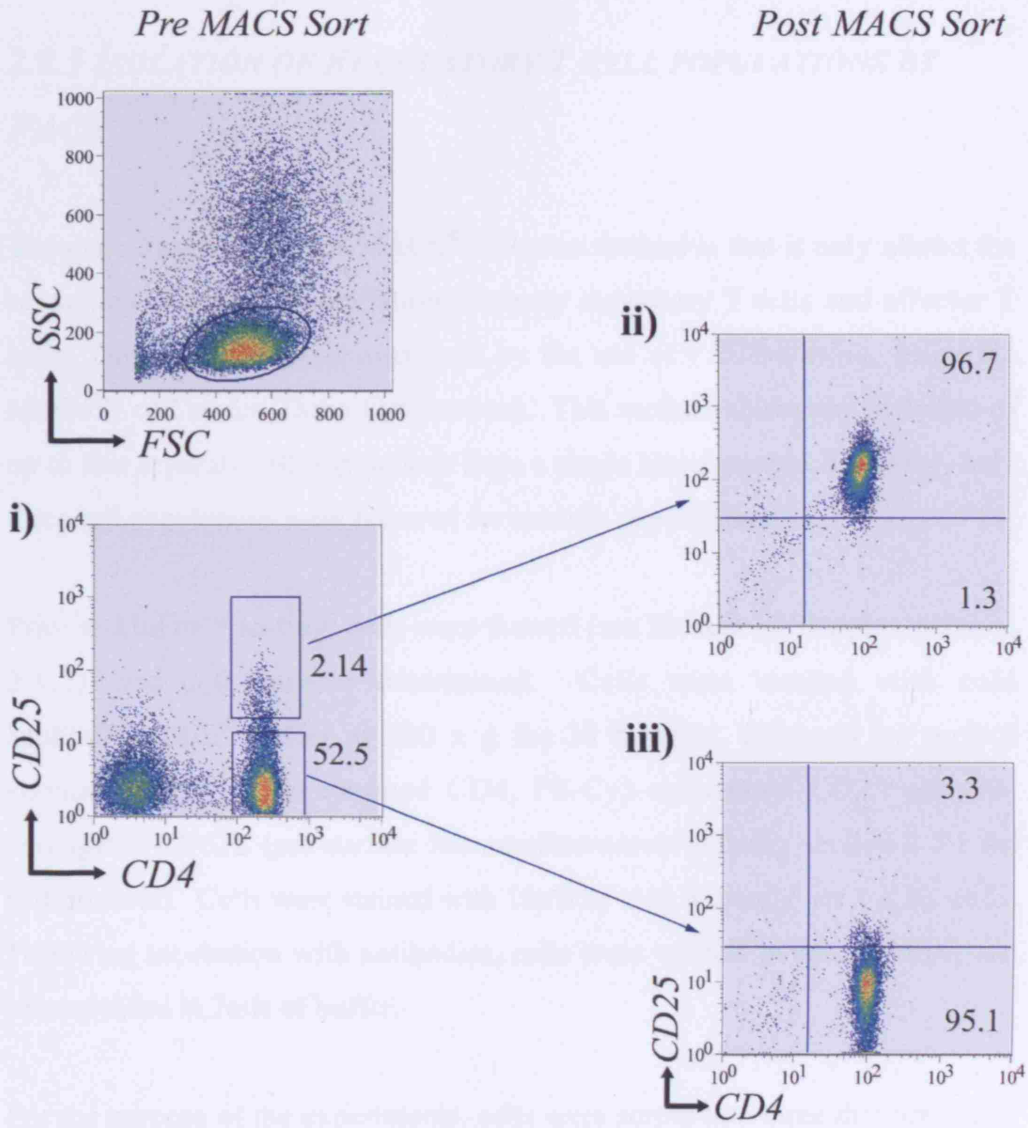


FIGURE 2.2.2 PURITY OF MACS®-ISOLATED REGULATORY T CELLS

PBMC from health controls and active RA patients were labelled with magnetic beads, as described in section 2.2.2. Labelled cells were initially passed through LD MACS® columns to deplete all non $CD4^+$ T cells. Unlabelled $CD4^+$ T cells were subsequently passed through LS MACS® columns in order to obtain $CD4^+CD25^+$ T cells. (i) shows the percentage of $CD4^+CD25^+$ regulatory T cells from PBMC population prior to MACS® isolation. (ii) and (iii) show representative FACS plots of percentage purity of $CD4^+CD25^+$ and $CD4^+CD25^-$, respectively.

2.2.3 ISOLATION OF REGULATORY T CELL POPULATIONS BY FACS

The main limitation of the MACS®-isolation method is that it only allows the separation of two cell populations, namely regulatory T cells and effector T cells. This limitation was overcome by the use of FACS-sorting, using the MoFlo™ cell sorter (Dako Cytomation). This method allows the isolation of up to four separate cell populations from a single blood sample. However, only three cell populations were required for specific experiments.

Prior to MoFlo™ sorting, cells were thawed (see Storage of Samples, section, 2.1.2), and cell number determined. Cells were washed with cold MoFlo™/MACS® buffer at 420 x g for 10 minutes, followed by surface staining with FITC-conjugated CD4, PE-Cy5-conjugated CD25 and PE-conjugated CD62L (see surface immunofluorescent staining section 2.5.1 for full protocol). Cells were stained with 10µls of each antibody per 1 $X10^7$ cells. Following incubation with antibodies, cells were washed twice as above, and re-suspended in 2mls of buffer.

For the purpose of the experiments, cells were sorted into three distinct T cell populations:

1. $CD4^+CD25^-$ T effectors

2. $CD4^+CD25^{hi}CD62L^+$ T regs

3. $CD4^+CD25^{hi}CD62L^-$ T regs

Figure 2.2.3 illustrates the gating strategy used to isolate the above three population of T cells. Purity of cell populations was characteristically greater than 95%. Representative purity FACS plots can also be seen in figure 2.2.3.

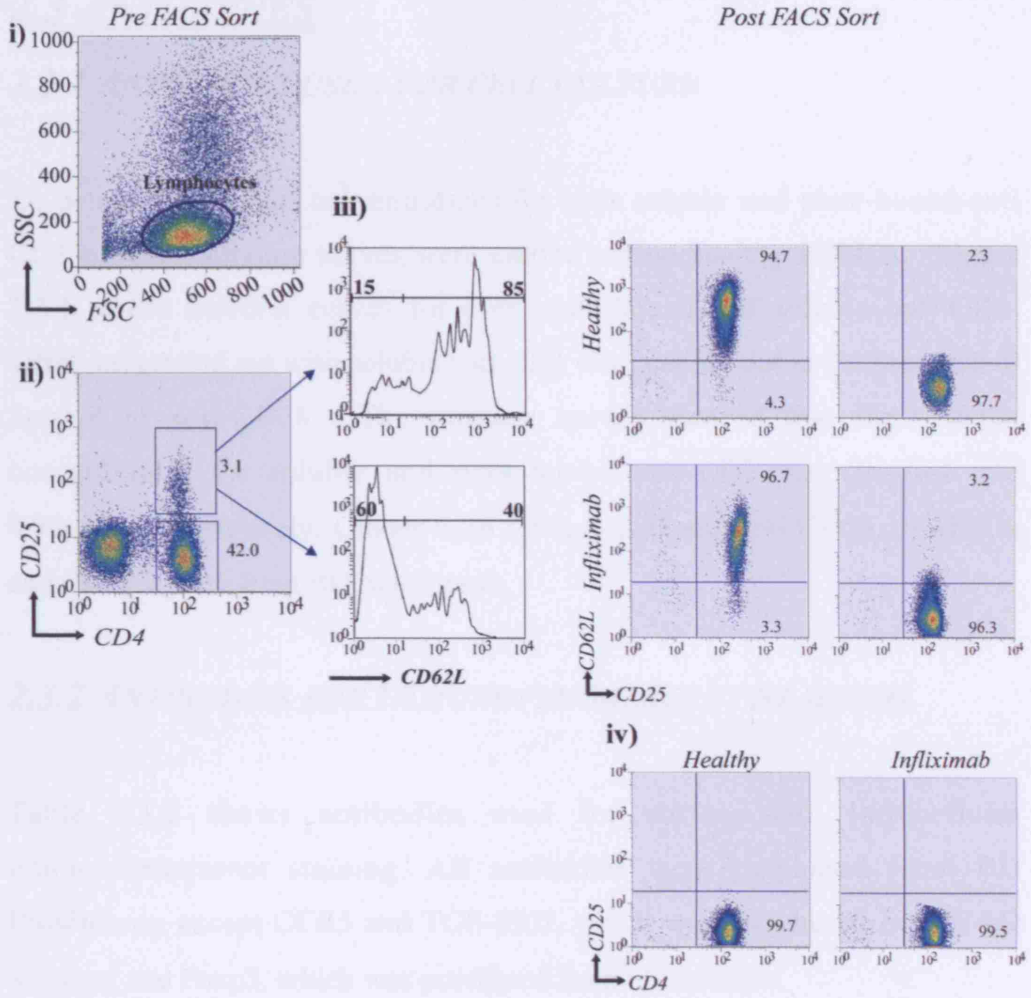


FIGURE 2.2.3 PURITY OF FACS-ISOLATED REGULATORY T CELLS

PBMC from controls and patients were initially surface stained with conjugated anti-human antibodies (CD4, CD25 and CD62L), as described in section 2.2.3 Cells were gated on the lymphocyte population (i), and then sorted on the total $CD4^+$ T cell population, according to the $CD4^+CD25^-$ and $CD4^+CD25^{hi}$ populations. The latter cell population was further sorted into two populations: $CD62L^+$ and $CD62L^-$ fractions. (ii) depicts the gating for $CD4^+CD25^-$ and $CD4^+CD25^-$ on total CD4. (iii) Representative histograms shows proportion of $CD4^+CD25^{hi}CD62L^+$ T cells in healthy and infliximab-treated patients; dot plots show representative percentage purities. (iv) shows representative purities of $CD4^+CD25^-$ fractions.

2.3 ANTIBODIES

2.3.1 ANTIBODIES USED FOR CELL CULTURE

To determine optimal concentrations for both soluble and plate-bound-anti CD3 titration, titration curves were carried out on healthy PBMCs. Figure 2.3.1 shows titration curves for both plate-bound and soluble anti-CD3. Titrations carried out with soluble anti-CD3 were carried out in the presence of 2μg/ml of anti-CD28. The titration curves showed that the optimal concentration for soluble and plate-bound anti-CD3 was 2μg/ml and 0.75μg/ml, respectively. Clones used for anti-CD3 and anti-CD28 are Hit-3a and CD28.2, both from BD biosciences.

2.3.2 ANTIBODIES FOR IMMUNOFLUORESCENT STAINING

Table 2.3.2 shows antibodies used for surface and intracellular immunofluorescent staining. All antibodies were purchased from BD Biosciences, except CCR5 and TGF-βRII, which were purchased from R&D Systems, and Foxp3, which was purchased from eBioscience.

2.3.3 ANTIBODIES FOR NEUTRALISATION ASSAYS & WESTERNS

Anti-TGF-β1 (9016.2) and anti-human IL-10 (25209) were used, both purchased from R&D systems. Isotype controls (IgG1 and $IgG2_B$), were also purchased from R&D Systems. Concentrations of neutralisation antibodies are shown in figure legends. Rabbit anti-phospho-Smad 2 and rabbit anti-Smad 2 (for western blot analysis), was purchased from Zymed Laboratories (part of Invitrogen Inc.), used at 2μg/ml. Goat anti-rabbit secondary antibody (Sigma) was used at a 1:10000 dilution. Dilution in blocking buffer, see appendix II.

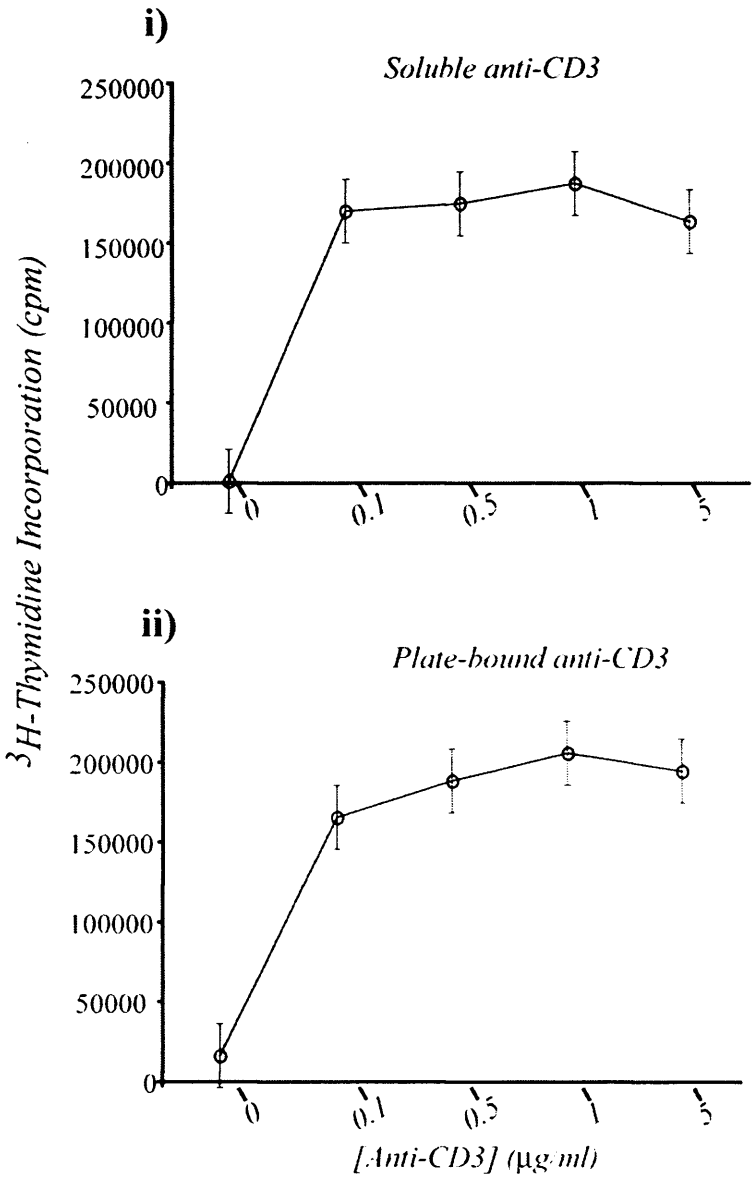


FIGURE 2.3.1 DETERMINATION OF OPTIMAL SOLUBLE AND PLATE-BOUND ANTI-CD3 CONCENTRATION

Healthy PBMC were cultured for five days in complete medium at 37°C, 5%CO_2, and thymidine added in the last 18 hours of culture to measure proliferation. (i) PBMCs were cultured with 2µg/ml soluble anti-CD28 in the presence of increasing concentration of soluble anti-CD3 (0.1, 0.5, 1 and 5µg/ml). (ii) Prior to culturing PBMC, plates were coated overnight at 4°C with increasing concentrations of plate-bound anti CD3 (0, 0.1, 0.5, and 5µg/ml). Results shown as counts per minute (cpm) and are means of six values. Error bars represent SEM.

Antibody	Colour	Clone	Isotype	Type of staining	Company
CD4	APC	RPA-T4	Mouse IgG1κ	Surface	BD Pharmingen™
	FITC				
	PE				
CD25	PE-Cy5	M-A251	Mouse IgG1κ	Surface	BD Pharmingen™
CD62L	PE	Dreg-56	Mouse IgG1κ	Surface	BD Pharmingen™
CCR5 (CD195	FITC	45502	Mouse IgG2Bκ	Surface	R & D Systems
	PE-Cy7	2D7/CCR5	Mouse IgG2Bκ		BD Pharmingen™
CD40L (CD154)	APC	TRAP1	Mouse IgG1κ	Surface	BD Pharmingen™
CTLA-4 (CD152)	PE	BN13	Mouse IgG2a	Intracellular	BD Pharmingen™
	Biotinylated				
Foxp3	PE	PCH-101	Rat IgG2a	Intracellular	eBioscience
	Alexafluor-488				
TNF-α	PE	Mab11	Mouse IgG1κ	Intracellular	BD Pharmingen™
	PE-Cy7				
IFN-γ	PE-Cy7	4S.B3	Mouse IgG1κ	Intracellular	BD Pharmingen™
IL-10	PE	JES3-19F1	Rat IgG2a	Intracellular	BD Pharmingen™
	APC				

TABLE 2.3.2 ANTIBODIES USED FOR IMMUNOFLUORESCENT STAINING

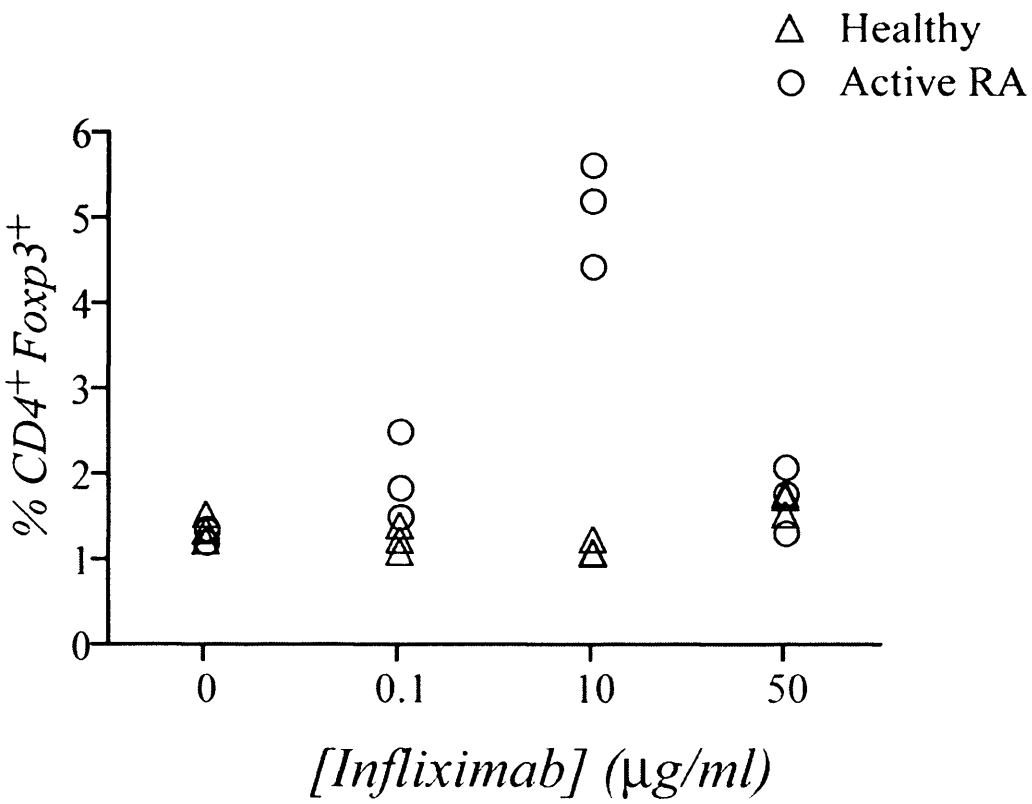


FIGURE 2.3.3 DETERMINATION OF OPTIMAL INFLIXIMAB CONCENTRATION FOR IN VITRO CULTURE

T effectors from three healthy controls and three active RA patients were isolated via MACS®. Cells were stimulated with 2μg/ml soluble anti-CD3 and 2μg/ml anti CD28, and cultured for 24 hours in the presence of increasing infliximab concentration (0, 0.1, 10 and 50μg/ml). Following culture, cells were surface stained for CD4 and intracellularly stained for Foxp3. Results are means of three healthy controls and three active RA patients. Error bars represent SEM

For neutralisation experiments, anti-TNF-α (infliximab, Schering-Plough) – figure 2.3.3 shows titration curve for optimal infliximab concentration for *in*

vitro experiments. To determine the optimal infliximab concentration T effectors from healthy controls and active RA patients were stimulated for 24 hours with 2μg/ml of anti-CD3 and anti-CD28 plus infliximab (0.1, 10, and 50μg/ml), using Foxp3 as a read-out. The graph in figure 2.3.3 shows that the optimal infliximab concentration is at 10μg/ml, inducing around 5% Foxp3 in active RA T effectors, but not healthy T effectors. An interesting observation with active RA T effectors is at 50μg/ml of infliximab, with the percentage of $Foxp3^+$ cells falling to 1.5%. One explanation for this could be that a high concentration, infliximab is inducing apoptosis of the T cells. This mechanism of action for infliximab has previously been shown in infliximab-treated Crohn's patients (245, 251).

2.4 CELL CULTURE

For all cell culture experiments, cells were cultured in 96-well U-bottomed plates (Nunc) at a concentration of 2 x 10^5 cells per ml in complete medium: RPMI-1640 supplemented with 100U/μg/ml penicillin / streptomycin (Life Technologies) and 10% FCS, at 37°C, 5%CO_2.

CELL CULTURE FOR PROLIFERATION ASSAYS

Cells were cultured for a total of 5 days, and pulsed with 1μCi/well tritiated thymidine ([^{3}H] Tdr) (MP Biomedicals) in the final 18 hours. Cells were harvested onto filter mats (Perkin Elmer; Tometec 2 harvester, Wallace), and proliferation was measured using a liquid scintillation counter.

INTRACELLULAR/ELISA DETECTION OF CYTOKINE

For intracellular detection of cytokines, cells were cultured for 48 hours. In the final 5 hours of culture, cells were stimulated with 50ng/ml PMA and 250ng/ml ionomycin with 1μl/ml Golgi Plug™ (see section 2.5.2 for full intracellular staining protocol).

For detection of cytokines TGF-β and IL-17 by ELISA, cells were stimulated for 72 hours.

2.5 FLOW CYTOMETRIC ANALYSIS

For both surface and intracellular staining procedures, staining was carried out in 96-well plates or directly in 5ml polypropolene (FACS) tubes. Volumes of staining buffers were adjusted accordingly: In 96-well plates, 150µls of buffer used, and in 5ml FACS tubes, 500µls of buffer used. All buffers used for intracellular staining were purchased from BD Biosciences.

2.5.1 SURFACE IMMUNOFLUORESCENT STAINING

4 x 10^4 cells per sample were washed twice at room temperature in FACS buffer at 420 x g for 10 minutes. Once spun down, the buffer was discarded, and cell pellet re-suspended. Cells were stained with relevant conjugated anti-human antibodies (see section 2.3.2.2.1) and incubated for 15 minutes at room temperature in the dark. Following the incubation period, cells were washed twice as above, and then fixed with 2% paraformaldehyde (2g in 100mls of 1 x PBS) for 15 minutes at 4°C. Cells were then washed twice, re-suspended in 200 µls of FACS buffer and transferred to FACS tubes, ready for acquisition (see section 2.5.3).

2.5.2 INTRACELLULAR IMMUNOFLUORESCENT STAINING

2.5.2.1 INTRACELLULAR STAINING FOR THE DETECTION OF CYTOKINES

Cells were initially surface stained as above, followed by the addition of 50µls of Cytofix™ for 20 minutes at 4°C. Cells were then washed twice (420 x g, 5 minutes) with 1 X Permwash™ (10 X Permwash™ in dH_20). Prior to the second wash, cells were left in Permwash™ for 5 minutes to facilitate permibilisation of cell membranes. Relevant conjugated anti-human antibodies were added and cells were incubated for 15 minutes at 4°C in the dark, washed twice as above and washed for a final time in FACS buffer. Cells were re-suspended in 200mls of FACS buffer, transferred to FACS tubes, ready for

acquisition (see section 2.5.3).

2.5.2.2 INTRACELLULAR STAINING FOR FOXP3

Cells were initially surface stained as described in section 2.5.1. However, cells were not fixed with 2% paraformaldehyde. Cells were fixed (100µls/ well or 1ml/ tube) in freshly prepared fixation / permeabilisation buffer, made up according to manufacturer's guidelines (eBioscience). It is important that as soon as the fixation / permeabilisation buffer is added to the cells, that cells are either gently pulse vortexed, or re-suspended to ensure even distribution of buffer. Cells were fixed for 30 minutes in the dark at 4°C. Following incubation, cells were washed once with cold FACS buffer (100µls/well or 1ml/tube) at 400 X g for 5 minutes. Cells were then washed twice in 1 X permeabilisation buffer (eBioscience) (200µls/well or 2mls/ tube) and centrifuged at 400 X g for 5 minutes.

Following washings with 1 X permeabilisation buffer, cells were blocked with 2% normal rat serum (i.e. 2µls serum in 100µs final volume). Cells were incubated for 15 minutes in the dark at 4°C. Without washing, 5µls of Foxp3 antibody was added to the cells after the 15 minute incubation. Cells were incubated for a f permeabilisation buffer for a further 30 minutes in the dark at 4°C. Cells were washed twice with 1 X permeabilisation buffer, and washed once in cold FACS buffer.

2.5.3 ACQUISITION OF SAMPLES

Cells were acquired on the BDLSR. Prior to acquiring samples, compensation was carried out, to remove any spill-over of fluorescence that may be present in the wrong channel (this occurs because emission spectra of antibody fluorescent labels overlap). Compensation was carried out using single stain controls. Settings were saved each time, so that these settings could be used for similar experiments, using the same cell types. During acquisition, cells were gated on the lymphocyte population of the SSC/FSC plot (see figure 2.5), and 10000 events were saved within the gated region.

2.5.4 ANALYSIS OF ACQUIRED DATA

2.5.4.1 ANALYSIS OF FACS DATA

Analyses of FACS data were carried out using FlowJo software (Treestar Inc.). Figure 2.5 shows gating strategies for the analysis of the four main populations investigated: $CD4^+CD25^-$, $CD4^+CD25^{hi}$, $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells. Briefly, the lymphocyte gate was chosen according to the forward/side scatter (FSC/SSC) plot (figure 2.5 i). For analysis of $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ populations, cells were gated within the total $CD4^+$ population and the top 3-4% cells were gated on to obtain the $CD4^+CD25^{hi}$ Treg population. Cells were gated within the 1st log to obtain the $CD4^+CD25^-$ T effector population (figure 2.5 ii). For analysis of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ populations, cells were initially gated on the total $CD4^+$ population. A Foxp3 isotype control was used to determine where to place quadrants to obtain both Foxp3 populations (figure 2.5 iii). All gates and quadrants where copied and pasted into each sample to ensure equal analyses.

2.5.4.2 STATISTICAL ANALYSES

Statistical analyses were carried out using Prism software. Since it was necessary to compare patient groups (i.e. active RA or infliximab-treated patients) with healthy controls, a two-tailed, one-sample (unpaired) student's T test (95% significance value) was used to determine any statistical difference between controls and patients.

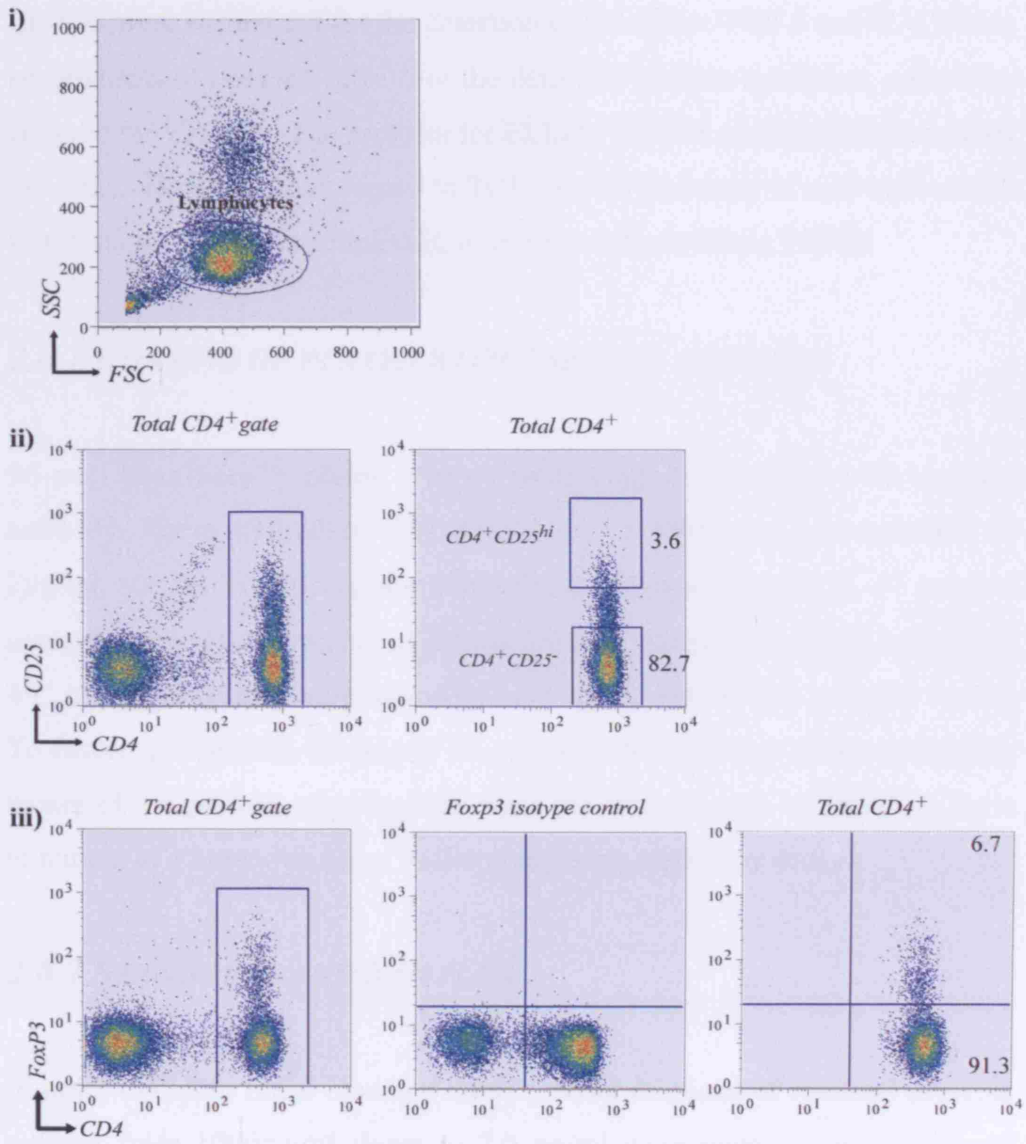


FIGURE 2.5 GATING STRATEGIES FOR ANALYSES OF TREG AND T EFFECTOR POPULATIONS

Cells were gated on the lymphocyte population according to the forward/side scatter (FSC/SSC) plot (i). Cells were then gated on the total $CD4^+$ population in order to obtain $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ populations (ii). Additionally, quadrants where determined for Foxp3 populations according to the Foxp3 isotype control (iii).

2.6 ELISAs

ELISAs were carried out for the detection of cytokines, TGF-β and IL-17 from supernatants of cultured cells. For the detection of these cytokines, cells were cultured for 72 hours. The protocol for ELISAs for both cytokines is essentially the same. However, with regard to TGF-, it was necessary to acid-activate the supernatant, `and then neutralise it, in order to activate latent TGF-β.

2.6.1 COATING OF PLATES WITH CAPTURE ANTIBODY

96-well MaxiSorp™ plates (Nunc) were coated overnight with capture antibody. These antibodies were diluted in 1 X PBS, at a concentration of 2μg/ml, for the TGF-β capture antibody, and 4μg/ml for the IL-17 capture antibody, with a final volume of 100μls per well. Plates were sealed, and left at 4°C overnight. Plates were then washed 5 times with 300μls of wash buffer. To ensure non-specific binding of the antibody did not occur (due to the sticky nature of the plates), coated plates were blocked at room temperature for a minimum of 1 hour with assay buffer plates were washed as above.

2.6.2 STANDARDS AND SAMPLES

In order to obtain linear standard curves, serial dilutions of standard proteins ranging from 1000pg/ml down to 7.5 pg/ml were done. Supernatants of samples were added neat to wells (50μls/well). With regard to TGF-β detection, samples must be acid-activated, and the neutralised, before being plated: To acid-activate, 20μls of 1N HCL was added to 100μls of supernatant for 10 minutes at room temperature. To neutralise, 40μls of 1.2N NaoH/0.5M HEPES was added to supernatants, mixed thoroughly, and then plated out.
All standards and samples were done in duplicate. Plates were incubated at room temperature for 3 hours. Plates were then washed as above.

2.6.3 DETECTION ANTIBODIES

Biotin-conjugated antibodies were diluted in assay buffer at a 1:1000 concentration, with a final volume of 100µls added to each well (including wells that did not contain samples). Plates were covered and incubated at room temperature for 2 hours, followed by washing, as above. Streptavidin-horseradish peroxidase (HRP) was diluted 1:5000 in assay buffer, and 100µls added to each well (including wells that did not contain samples). Plates were incubated at room temperature on a microplate shaker (set at 200 rpm), for 1 hour and then washed. Prior to adding TMB substrate solution, it must be brought to room temperature. 100µls of substrate solution was added to each well, incubated in the dark for 10 minutes, or until the lowest standard concentration is the same colour as a blank well. The enzyme-substrate reaction was then stopped quickly by adding 100µls of stop solution (concentrated H_2S0_4 in dH_20). Plates were tapped gently, to ensure thorough mixing of stop solution.

2.6.4 CALCULATION OF PROTEIN VALUES

Optical densities (OD) of each well determined within 30 minutes using a micro-plate reader (Anthos) set at 450nm. The amount of protein in each sample was calculated by using Prism software, using the X and Y template. In column "X" values of standards were added (i.e. highest standard concentration is equivalent to a value of 1000 pg/ml protein). In the "Y" columns values from corresponding OD values were added, followed by OD values of samples. Using the standard curve (see figure 2.6.4), interpolated X values were calculated (via Prism software), in order to obtain protein concentrations of samples.

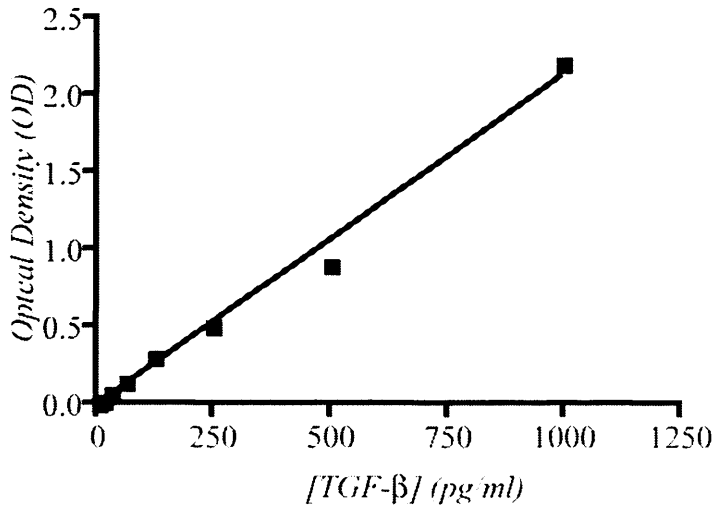


FIGURE 2.6.4 STANDARD CURVE FOR DETERMINATION OF PROTEIN CONCENTRATIONS

Standard curves are used in ELISAs and western blot analysis to determine the protein concentration of a given sample. By using protein standards, standard curves can be used to interpolate protein concentration. For example, using the graph above, if the OD of a sample is 0.5, its protein concentration is around 250pg/ml.

2.7 WESTERN BLOT ANALYSIS

Full recipes for all buffers used for western blot analysis are given in appendix II

2.7.1 PREPARATION OF CELL LYSATES

Cells were washed three times in cold 1XPBS in a micro-centrifuge (Eppendorf) (micro-centrifuge was pre-cooled prior to use) at 14000 rpm at 4°C for 5 minutes. After first and second wash PBS was decanted and cell pellet was re-suspended in PBS. After the final wash, PBS was carefully decanted, without disturbing the cell pellet. 1 $x10^6$ cells were re-suspended in 50µls of cell lysis buffer. To avoid clumping of cells, cells were kept on ice. To ensure even distribution of cell lysis buffer, and to avoid clumps, cells were pipetted up and down a few times with the lysis buffer. As soon as cells were lysed, they were transferred to -80°C, until required for protein assay and western blot analysis.

2.7.2 PROTEIN ASSAY

In order to determine how much protein was present within each sample, protein assays were carried out. Frozen cell lysate samples were thawed and spun down at 14000 rpm at 4°C for 10 minutes. Samples were diluted in 1 X PBS in a 1/3 dilution (i.e. 10µls of sample in 20µls of 1 X PBS). 10µls of pre-prepared standard (A-I) (Pierce, Perbio) were added in succession to 96-well MaxiSorp™ plates (Nunc), followed by 10µls of diluted samples. Depending on how much sample was available, samples were plated out either in duplicate or triplicate, so that mean values could be calculated. Once samples were plated out, 200µls of Pierce reagent mix (Reagent A: Reagent B 50:1) (Pierce, Perbio) was added to all wells containing samples, including standards. The protein assay plate was then left to incubate at 37°C for 30 minutes. Following incubation, optical densities were determined by reading standards and samples in a micro-plate reader at a wavelength of 595nm.

2.7.2.1 CALCULATION OF AMOUNT OF PROTEIN IN SAMPLE AND HOW MUCH SAMPLE TO LOAD

The amount of protein in each sample was calculated by using Prism software, using the X and Y template. In column "X" values of protein standards were added (i.e. protein standard "A" is equivalent to a value of 2000 mg/ml protein). In the "Y" columns values from corresponding OD values were added, followed by OD values of samples. Using the standard curve, interpolated X values were calculated (via Prism software), in order to obtain protein concentrations of samples.

To calculate the amount of sample that could be loaded for western blot, the optimal amount of protein to be loaded was determined. This is usually between 10 and 15μg. Westerns carried out in this PhD, an optimal amount of 10μg was used. Since protein concentration values are in mg/ml, and optimal protein amount is in μg, protein concentration values must be divided by 1000 to convert them into μg/μl. Once protein concentrations are converted into μg/μl, the amount of protein (x) that can be loaded per well can be calculated:

For an optimal protein amount of 10μg

$$x = 10\mu g \div \text{protein concentration } (\mu g/\mu l)$$

If the original protein assay was done in a 1 in 3 dilution, then protein values were divided into 3 for calculation. It should also be noted that the maximum volume that can be loaded is 30μls, and 5μls of sample buffer is always added. Therefore, maximum sample volume that can be added is 25μls.

2.7.3 IMMUNOBLOTTING

2.7.3.1 SETTING UP RESOLVING AND STACKING GELS

The percentage resolving gel depends on the size of the protein of interest. Typically, a 10% gel is ideal for proteins that are between 25-50 kDa.

To set up gel running kit (Scie-Plas), glass plates were washed first with water, then with methanol. Spacers were placed onto glass plates and then set up in gel unit, tightened, and placed on casting base.

Resolving gels were poured carefully, using a 10 ml pipette, and constantly checked for any leakage. A 2 cm gap was left at the top. The gel was covered with butan-1-ol. This keeps the gel moist and levels the gel at the top. A small amount resolving gel was left in the tube to make sure gel polymerised. Gels were left to set for 1 hour at room temperature.

Once the resolving gel had set, the butan-1-ol was washed off and the top of the gel was carefully dried with blotting paper. Stacking gel was prepared, as described in appendix II. Prior to adding stacking gel, combs were put into place (the combs sets out the lanes for loading samples). Stacking gel was carefully added, ensuring no bubbles were made, and left to set for 30 minutes at room temperature.

2.3.7.2 PREPARATION AND LOADING OF SAMPLES

5µls of sample buffer was added to each sample, including the molecular weight marker, and heated for 5 minutes at 100°C. Samples spun down at 14000 rpm for 40 seconds.

Following setting of stacking gel, combs were removed swiftly, to minimise any damage to lanes. Prior to loading lanes, the gel unit was placed into the gel tank and half filled with running buffer. Gels were flushed with running buffer

to prevent drying out whilst loading samples.

The first and last lanes were left blank (loaded with sample buffer only), and the second lane was loaded with molecular weight marker. Samples were carefully loaded into subsequent lanes, and any remaining blank lanes were loaded with sample buffer.

2.3.7.3 RUNNING AND TRANSFER OF GELS

RUNNING THE GEL

It is important that the running buffer is filled to the top of the gel tank, as insufficient running buffer prevents proper running of proteins on the gel. Proteins were run at 120 volts for 1.5-2 hours (or when samples have reached the bottom if the glass plates). The lower voltage combined with the longer time prevents "smiling" of the gel. This is when the proteins run unevenly, and the outside lanes get distorted.

TRANSFER OF PROTEINS TO MEMBRANE

Prior to running gels, 8 X 8 cm PVDF membranes (Millipore) were activated in methanol for 15 seconds, and then transferred to distilled water. Membranes were agitated in water until the oily sheen disappeared.

Activated membranes were then left to soak, together with blotting paper and sponges from the gel running kit, in cold transfer buffer (see appendix II) until required for transfer stage.

It should be noted that proteins are negatively charged, and therefore run from negative to positive. Hence, it is important the transfer "sandwich" is constructed properly. For the Scie-Plas kits the "sandwich" is made as follows:

1. Black frame
2. Sponge
3. Blotting paper
4. Gel
5. Blotting paper
6. Sponge
7. White frame

When placing the "sandwich" into the gel unit, the white frame must face positive to ensure proper transfer of proteins. Proteins were transferred at 200 mA for 1 hour.

2.3.7.4 BLOCKING AND APPLYING ANTIBODIES TO MEMBRANES

In order to prevent non-specific binding of antibody, membranes were incubated in blocking buffer for 1 hour at room temperature. Following blocking, membranes were incubated overnight, at 4°C in a 50 ml Falcon tube. Primary antibodies were diluted in blocking buffer at 1/500 – 1/1000 (final volume 5 mls), depending on primary antibody. **N.B** when probing for phosphorylated antibodies, 50 mMol of sodium fluoride (NaF) was added to the final concentration of the primary antibody. NaF is a phosphatase inhibitor, and therefore, prevents any degradation of phsophorylation.

Following overnight incubation in primary antibody, membranes were washed three times (three X 1 minute washes) in PBS-Tween at room temperature, and then incubated in the secondary antibody (1/10000 in blocking buffer). Membranes were incubate at room temperature for 1 hour, and then washed three times as above.

2.3.7.5 DEVELOPING MEMBRANES & CHECKING FOR EQUAL SAMPLE LOADING

Membranes were developed using Amersham standard detection reagents: 3mls of each reagent (reagent A and B) were mixed and added to the membranes. Membranes were agitated for 1 minute, and then assembled into the developing cassette. Once in the darkroom, membranes were exposed to developing film (Amersham), and developed initially for 1 minute. If nothing was seen on the film following 1 minute exposure, membranes were exposed for an additional 15 minutes, 1 hour or overnight to check for protein bands.

If membranes need to be re-probed for proteins of same/similar molecular weigh as original protein of interest, membranes must be stripped. Membranes were covered in Restore Western Stripping Buffer™ (Pierce, Perbio), covered with cling film, and incubated at 37°C for a maximum of 20 minutes. Membranes were exposed and films developed as described above, to check that all proteins have been successfully stripped from the membrane.

To check for equal loading of samples, membranes were probed overnight with GAPDH (1/10000 in blocking buffer), or for 1 hour at room temperature. Following incubation, membranes were washed three times as described above, and incubated for 1 hour at room temperature in secondary antibody (anti-mouse HRP for GAPDH 1/10000 in blocking buffer) and washed three times. Membranes were exposed and films developed as described above.

CHAPTER 3.1

REGULATORY T CELLS FROM ACTIVE RA PATIENTS ARE PHENOTYPICALLY ABNORMAL

3.1 RESULTS

Previous work done in this lab has shown that Tregs from patients with active disease are defective in terms of their inability to suppress T effector-derived pro-inflammatory cytokines (TNF-α and IFN-γ), a characteristic function of Tregs, but are still able to suppress T effector proliferation. In addition it had been shown that patients treated with the TNF-α-neutralising drug, infliximab, have a restored Treg function, where the suppressor cells are able to suppress pro-inflammatory cytokine production (255) .Tregs have been characterised by several activation markers, which include CCR5, CTLA-4 and CD62L (L-selectin). Additionally, other co-stimulatory molecules, including CD40L are involved in Treg immunoregulation.

To unravel the mechanism that underpin the defect in Tregs defective in patients with RA, the above markers were measured in Tregs from RA patients, and levels were compared to those expressed by Tregs from healthy controls and RA patients treated with infliximab. Moreover, due to the central role of TNF-α in RA pathology, the effect of this cytokine on the expression of these specific markers was investigated. In addition, because CD25 is also a marker of activation, it was important to investigate whether any changes in phenotype was due to increased activation, or if the effect was true for Tregs. Therefore, comparative phenotypic analysis was carried out using Foxp3 as an additional marker for Tregs.

Consequently, the work described in this chapter prompted the following two questions:

1. Do Tregs from active RA have an altered phenotype compared to healthy controls and patients treated with infliximab?
2. Since there is a restoration of function in Tregs from patients treated with infliximab, is TNF-α directly responsible for any phenotypic changes in RA Tregs?

3.1.1 FOXP3 EXPRESSION DOES NOT CHANGE IN ACTIVE RA PATIENTS OR IN HEALTHY TREGS IN THE PRESENCE OF TNF-α, BUT IS UP REGULATED IN INFLIXIMAB PATIENTS

The above phenotypic analysis of Tregs from active RA patients suggests why these cells might be defective within this patient group. Previous work from this lab has already demonstrated that there is no significant difference in Treg number from active RA patients, compared to controls, when based on the $CD4^{+}CD25^{hi}$ population (255). However, since Foxp3 is a Treg-specific marker, it was necessary to investigate whether any phenotypic and/or numerical differences, in terms of Foxp3, could be seen in Tregs from active RA patients.

PBMCs from healthy controls, active RA and infliximab patients were surface stained for CD4 and CD25, and intracellularly stained for Foxp3. PBMCs from etanercept patients were also stained for the above markers to compare Foxp3 numbers with healthy controls and to compare anti-soluble TNF-α therapy (etanercept) with anti-soluble and membrane TNF-α therapy (infliximab).

Analysis of Foxp3 expression in active RA patients confirmed previous findings of Treg numbers in this patient group. No statistical difference was observed in the percentage of $CD4^{+}Foxp3^{+}$ T cells in active RA patients (mean 4.7%) when compared to healthy controls (mean 5.2%) (figure 3.1.1.1). Similarly, no statistical difference in Foxp3 number was seen in RA patients treated with etanercept (mean 5.1%).

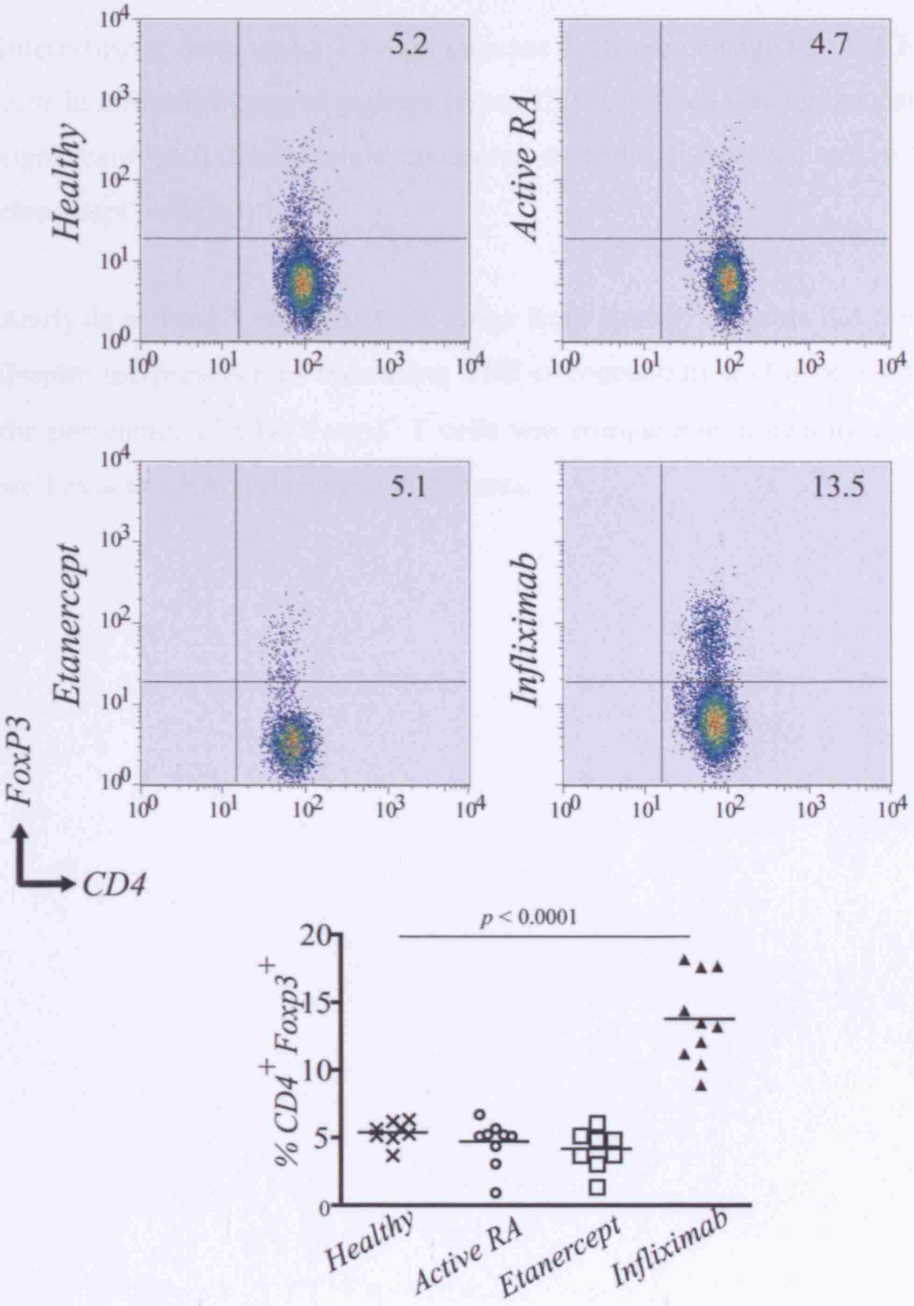


FIGURE 3.1.1.1 FOXP3 NUMBERS ARE NOT DECREASED IN ACTIVE RA PATIENTS, BUT ARE INCREASED IN INFLIXIMAB PATIENTS

PBMC from healthy controls, active RA, etanercept and infliximab patients were surface stained for CD4 and CD25, and intracellularly stained for Foxp3. Representative FACS plots shown. Cells were gated on $CD4^+Foxp3^+$ within the total $CD4^+$ population. (See figure 2.5 B in materials and methods for gating strategies)

Interestingly, there was a 2.5-fold increase in the percentage of $CD4^+Foxp3^+$ T cells in infliximab-treated patients (mean 13.5%), which was highly statistically significant ($p<0.0001$), when compared to healthy controls, active RA and etanercept patients.

Analysis of Foxp3 expression in Tregs from healthy controls did not change despite the presence of increasing TNF-α concentration (figure 3.1.1.2), and the percentage of $CD4^+Foxp3^+$ T cells was comparable to healthy controls, as well as active RA and etanercept patients.

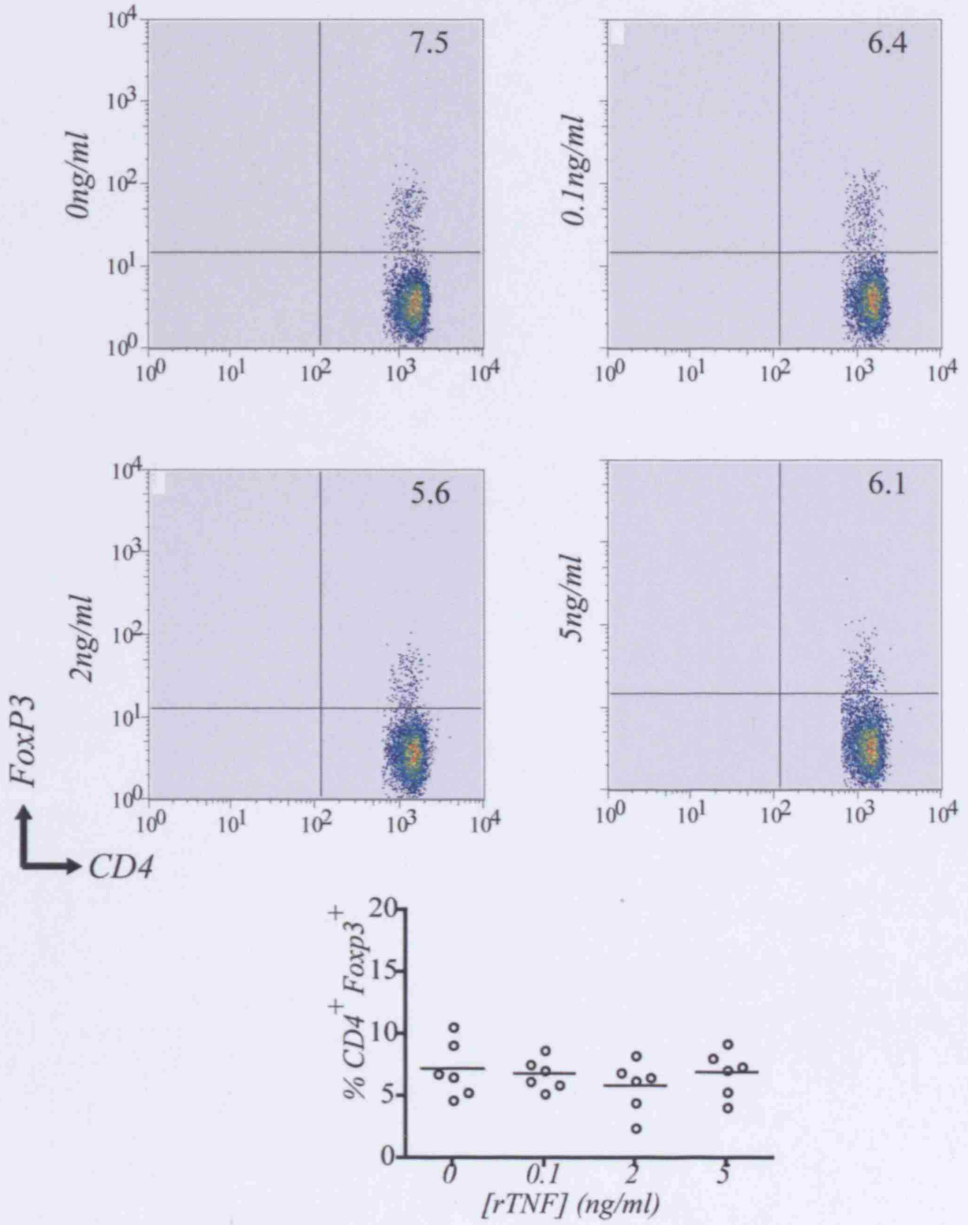


FIGURE 3.1.1.2 FOXP3 NUMBERS REMAIN UNCHANGED IN THE PRESENCE OF INCREASING CONCENTRATIONS OF RECOMBINANT TNF-α ON HEALTHY TREGS

PBMC from healthy controls were cultured for 24 hours with 0.75μg/ml plate bound anti-CD3 in the presence of increasing TNF-α concentration (0.1, 2 5ng/ml). Following culture, cells were stained and analysed as in 3.1.1.1.

3.1.2 CCR5 EXPRESSION IS DOWN REGULATED ON ACTIVE RA TREGS AND TNF-α REDUCES CCR5 EXPRESSION ON HEALTHY TREG

CCR5 is a chemokine receptor that has been found to have important implications in the ability of antigen presenting cells (APC) to recruit Tregs to sites of inflammation, and consequently, Tregs that lack CCR5 expression are unable to be recruited by APC (256). Numerous studies in CCR5-deficient mice, in addition to GVHD studies, have demonstrated enhanced cell-mediated immune responses during pathogen infection, and delayed-type hypersensitivity, all of which suggest an immunoregulatory role for CCR5.

Whole PBMC from healthy controls, active RA, and infliximab patients were stained (as described in material and methods) with CD4 and CD25, Foxp3 and CCR5. Cells were analysed by gating on $CD4^{+}CD25^{hi}$, $CD4^{+}Foxp3^{+}$ (Treg populations); and $CD4^{+}CD25^{-}$ and $CD4^{+}Foxp3^{-}$ (T effector populations) Analysis of healthy $CD4^{+}CD25^{hi}$ Tregs revealed that these cells expressed high levels of CCR5 on their surface (mean MFI = 70). These expression levels were similar to that seen on $CD4^{+}CD25^{hi}$ Tregs from infliximab-treated patients. However, Tregs from active RA patients expressed significantly lower ($p < 0.0001$) levels of this chemokine receptor (figure 3.1.2.1 i).

There was no significant difference between healthy controls, active RA and infliximab patients with respect to CCR5 MFI on $CD4^{+}CD25^{-}$ T effector cells. Interestingly, however, T effectors from active RA patients expressed higher levels of CCR5, compared with their Tregs (compare graphs in figure 3.1.2.1 i)

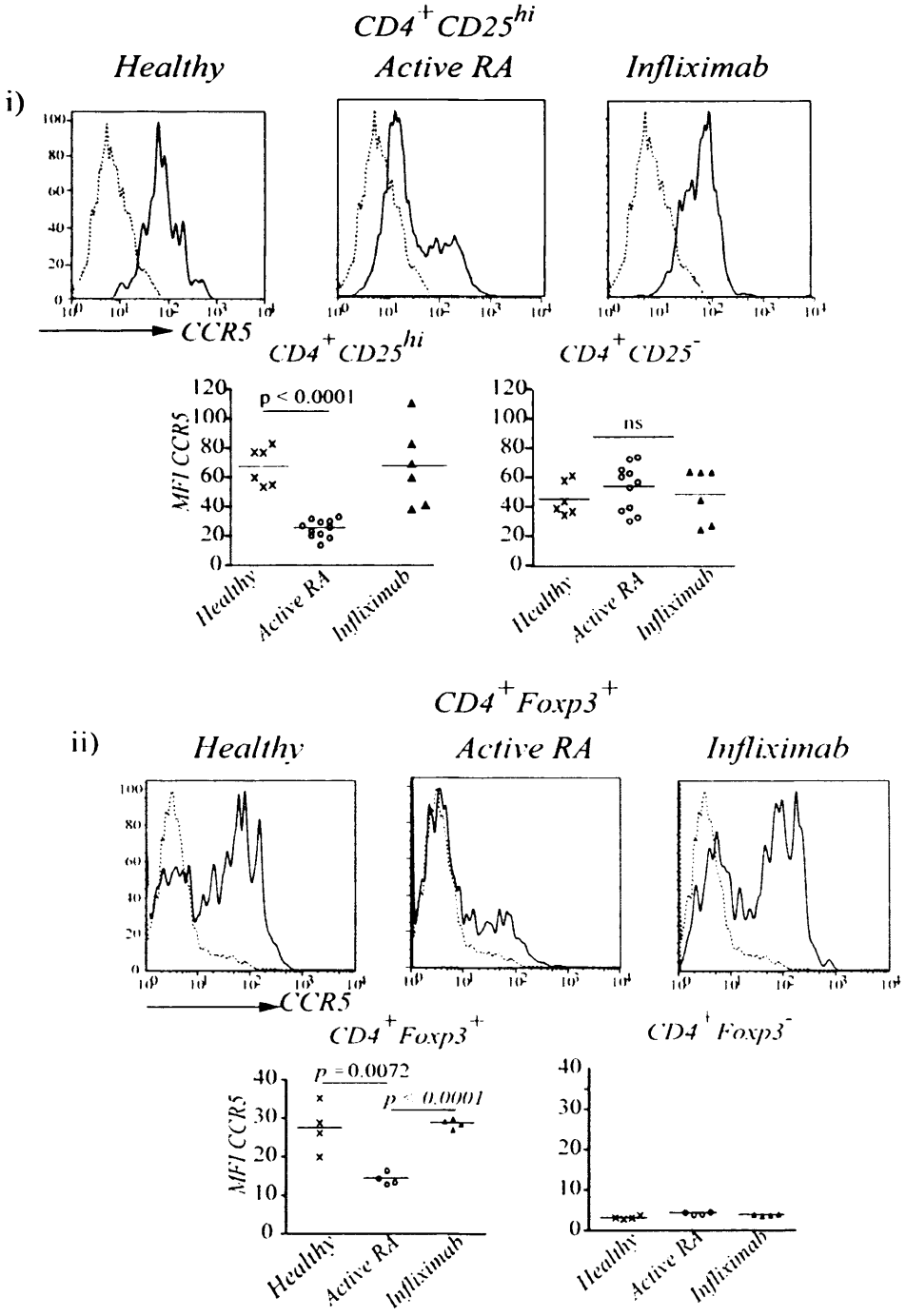


FIGURE 3.1.2.1 ACTIVE RA PATIENTS EXPRESS LOWER LEVELS OF CCR5 ON THEIR TREGS

PBMC from healthy controls, active RA and infliximab patients were surface stained ex vivo for CD4, CD25 and CCR5 (i). Additionally PBMC were intracellularly stained for Foxp3 (ii). Cells were gated on $CD25^{hi/-}$ or $Foxp3^{+/-}$ within the total $CD4^+$ T cell population Representative histogram plots shown; dotted lines illustrate isotype controls.

Analysis of CCR5 expression on $CD4^+Foxp3^+$ Tregs between healthy controls and active RA and infliximab patients demonstrated a similar expression pattern to that seen in $CD4^+CD25^{hi}$ Tregs: both healthy controls and infliximab patients expressed similar levels of CCR5 on their $CD4^+Foxp3^+$ Tregs (mean MFI 28 and 29, respectively). Active RA $CD4^+Foxp3^+$ Tregs expressed significantly lower levels ($p = 0.0072$), almost half the level compared to healthy controls (mean MFI 14.5). There was no significant difference in CCR5 MFI on $CD4^+Foxp3^-$ T effector populations, and unlike $CD4^+CD25^-$ T effectors, active RA $CD4^+Foxp3^-$ T effector did not express higher levels of CCR5 than their $CD4^+Foxp3^+$ counterparts (figure 3.1.2.1 ii).

Comparison of overall CCR5 levels between $CD4^+CD25^{hi}$ and $CD4^+Foxp3^+$ Tregs of each study group showed that CCR5 expression is higher on the former Treg population. For example, within the healthy control study group, mean CCR5 MFI is 70 for the $CD4^+CD25^{hi}$ Treg population, whereas for the $CD4^+Foxp3^+$ Treg population, the mean CCR5 MFI is 28. This difference in expression could be attributed to the fact that experiments looking at $CD4^+CD25^{hi}$ Treg population were carried out on different days to experiments looking the $CD4^+Foxp3^+$ Treg population, and this could explain the discrepancy of overall CCR5 expression between $CD4^+CD25^-$ and $CD4^+Foxp3^-$ T effector populations.

Whole PBMC from healthy controls and active RA patients were cultured for 24 hours with 0.75μg/ml plate-bound anti-CD3, together with increasing concentration of TNF-α (0, 0.1,2 and 5ng/ml). Following culture, cells were washed and stained for CD4 and CD25, and surface stained for CCR5.

Analysis of CCR5 expression on $CD4^+CD25^{hi}$ Tregs showed that even after 24 hour culture with plate-bound anti-CD3 alone, CCR5 expression were similar to that seen on *ex vivo* stained cells, where mean MFI for *ex vivo* and anti-CD3-stimulated Tregs, was 50 and 40, respectively

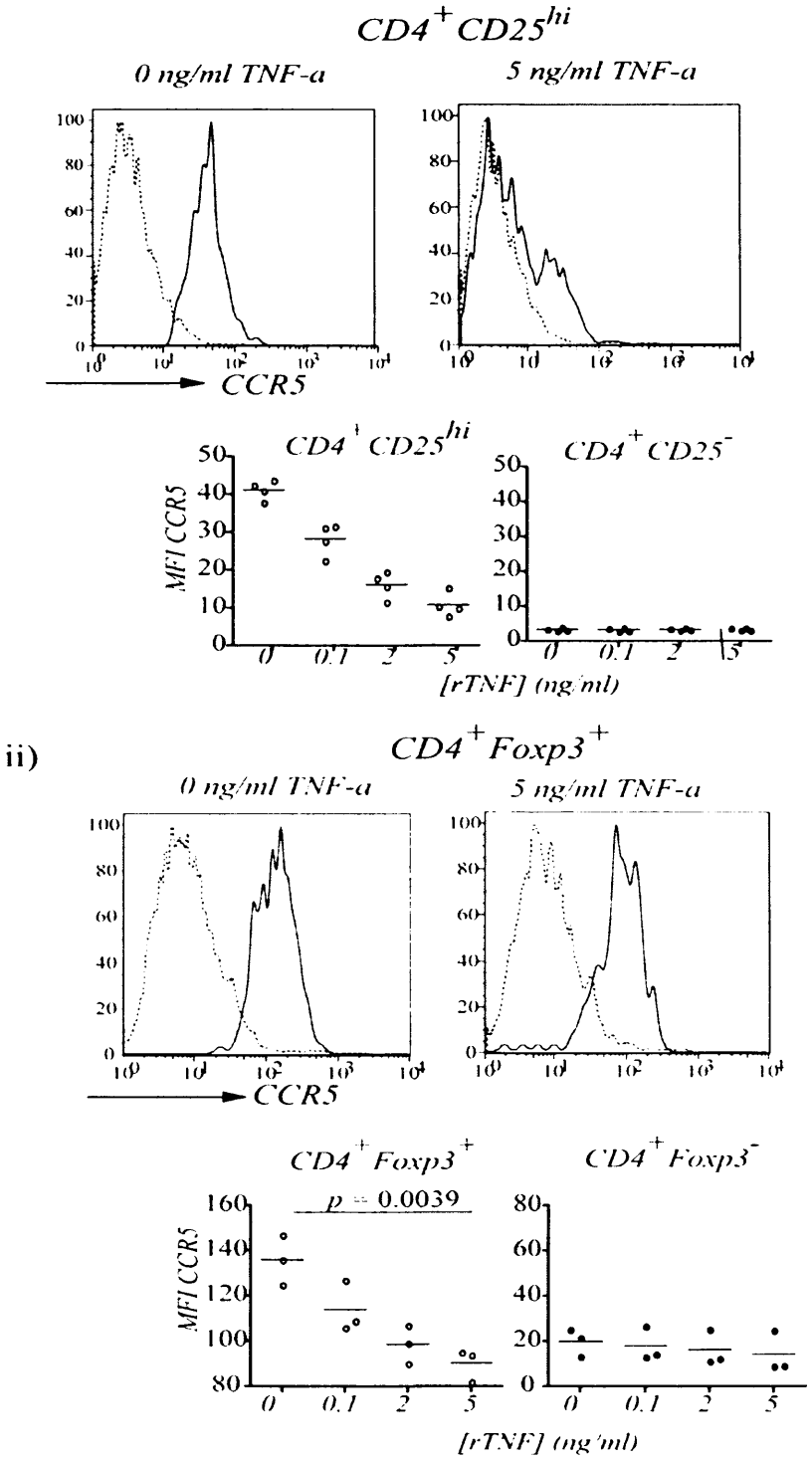


FIGURE 3.1.2.2 CCR5 EXPRESSION IS DOWN REGULATED ON HEALTHY TREGS IN THE PRESENCE OF TNF-α.

Healthy PBMC were cultured for 24 hours with 0.75μg/ml of plate-bound anti-CD3 in the presence of increasing levels of recombinant TNF-α (0, 0.1, 2 and 5ng/ml). Following culture, cells were stained and analysed as in 3.1.2.1

CCR5 expression was reduced in the presence of 0.1ng/ml of TNF-α when compared to anti-CD3 alone (see figure 3.1.2.2 i). This trend in reduced CCR5 expression continued in a dose-dependent fashion, with a significant reduction in expression at 5ng/ml of TNF-α ($p < 0.0001$). There was no significant difference, however, in CCR5 expression on $CD4^+CD25^-$ T effector cells despite increasing TNF-α concentrations, where expression remained lower than CCR5 MFI on Tregs at 5ng/ml TNF-α

Analysis of $CD4^+Foxp3^+$ Tregs revealed a similar pattern in a dose-dependent reduction of CCR5 MFI (figure 3.1.2.2 ii). However, mean MFIs were considerably higher at all TNF-α concentrations within the $CD4^+Foxp3^+$ Treg population when compared to the $CD4^+CD25^{hi}$ Treg population. For example, the mean CCR5 MFI in the absence of recombinant TNF-α was 140 within the $Foxp3^+$ population, compared to just 40 within the $CD4^+CD25^{hi}$ population. This suggests that on average there is more CCR5 expressed per cell on $CD4^+Foxp3^+$ Tregs following plate-bound anti-CD3 stimulation when compared to the $CD4^+CD25^{hi}$ population. However, it should be noted that Foxp3 expression/number did not increase following plate bound stimulation in the presence or absence of TNF-α (see figure 3.1.1.1). There was no significant difference in CCR5 MFI on $CD4^+Foxp3^-$ T effectors, in the presence of all given concentrations of TNF-α.

Comparison of mean CCR5 MFI for healthy Tregs in the absence of recombinant TNF-α (mean MFI 138) showed that this value is two-fold higher than the mean MFI of healthy Tregs *ex vivo* (mean MFI 70; figure 3.1.2.1). This difference in CCR5 MFI may be due to the fact that healthy Tregs in the absence of TNF-α were stimulated overnight, and this stimulation could have been responsible for the increased levels of CCR5.

3.1.3 CD40L EXPRESSION IS INCREASED ON TREGS FROM ACTIVE RA PATIENTS, AND IS UP-REGULATED ON HEALTHY TREGS IN THE PRESENCE OF TNF-α

The inhibition of co-stimulatory pathways between APC and responder T cells has been shown to be a mechanism by which Tregs exert their suppressor function. This has been demonstrated with the CD40-CD40L pathway, where the presence of CD40L on anergic T cells *in vitro* prevents the suppressive activity of these cells (257). The ability of CD40-CD40L interactions interfering with Treg function suggested that Tregs from active RA might actually over-express CD40L on their surface compared to healthy controls and infliximab-treated patients, thereby rendering them unable to suppress responder T cell effector functions. PBMC from healthy controls, active RA and infliximab treated patients were cultured for 4 hours with 250ng/ml ionomycin and 50ng/ml PMA in order to see CD40L expression, without influencing its expression, as would happen in the presence of anti-CD3 and anti-CD28 stimulation. Following culture, cells were stained as described in materials and methods for CD4, CD25 and CD40L.

Analysis of $CD4^+CD25^{hi}$ Tregs from healthy controls showed that CD40L MFI was extremely low (mean MFI 10), and was also low in $CD4^+CD25^{hi}$ Tregs from patients treated with infliximab (no significant difference between healthy controls and treated patients). However, there was a 5-fold increase in CD40L MFI on $CD4^+CD25^{hi}$ Tregs from active RA (mean MFI 55) compared to healthy controls and infliximab patients (see figure 3.1.3.1). There was no significant difference in CD40L expression on $CD4^+CD25^-$ T effectors between healthy controls, active RA and infliximab patients, and the three study groups expressed low levels of CD40L.

Analysis of CD40L in $CD4^+Foxp3^+$ Tregs revealed that there was no statistical difference in expression, between healthy controls and infliximab-treated patients. However, mean MFIs were 3-fold higher from $CD4^+Foxp3^+$ Tregs when compared to mean MFIs on $CD4^+CD25^{hi}$ Tregs (approximate mean MFI 30 in healthy and infliximab patients). Active RA $CD4^+Foxp3^+$ Tregs expressed significantly higher levels of CD40L (p=0.0002), compared to healthy controls. Interestingly, analysis of $CD4^+Foxp3^-$ T effectors revealed that healthy controls express much higher levels of CD40L, compared to their Tregs and active RA T effectors (p= 0.0015), whereas active RA and infliximab patients express much lower levels (figure 3.1.3.1 ii). The overall expression of CD40L expression on $CD4^+Foxp3^-$ T effectors is much higher than on $CD4^+CD25^-$ T effectors (compare figure 3.1.3.1 i and ii, right panels). This could be due to the fact that both experiments were carried out on different days.

CD40L expression on healthy $CD4^+CD25^{hi}$ Tregs stimulated with plate-bound anti-CD3 alone did not significantly differ when compared to *ex vivo* experiments (compare figure 3.1.3.1 i with figure 3.1.3.2 i). Similarly, the presence of 0.1ng/ml of TNF-α also had no effect on CD40L expression on Tregs. However, a significant increase in expression was observed when TNF-α concentration was increased to 2ng/ml (p = 0.0018), and the expression intensity of CD40L on Tregs in the presence of 5ng/ml of TNF-α, increased further, mirroring levels on active RA Tregs *ex vivo* (compare figure 3.13.1 i with figure 3.1.2.2 i). The expression of CD40L did not significantly differ with increasing TNF-α concentrations on $CD4^+CD25^-$ T effector cells.

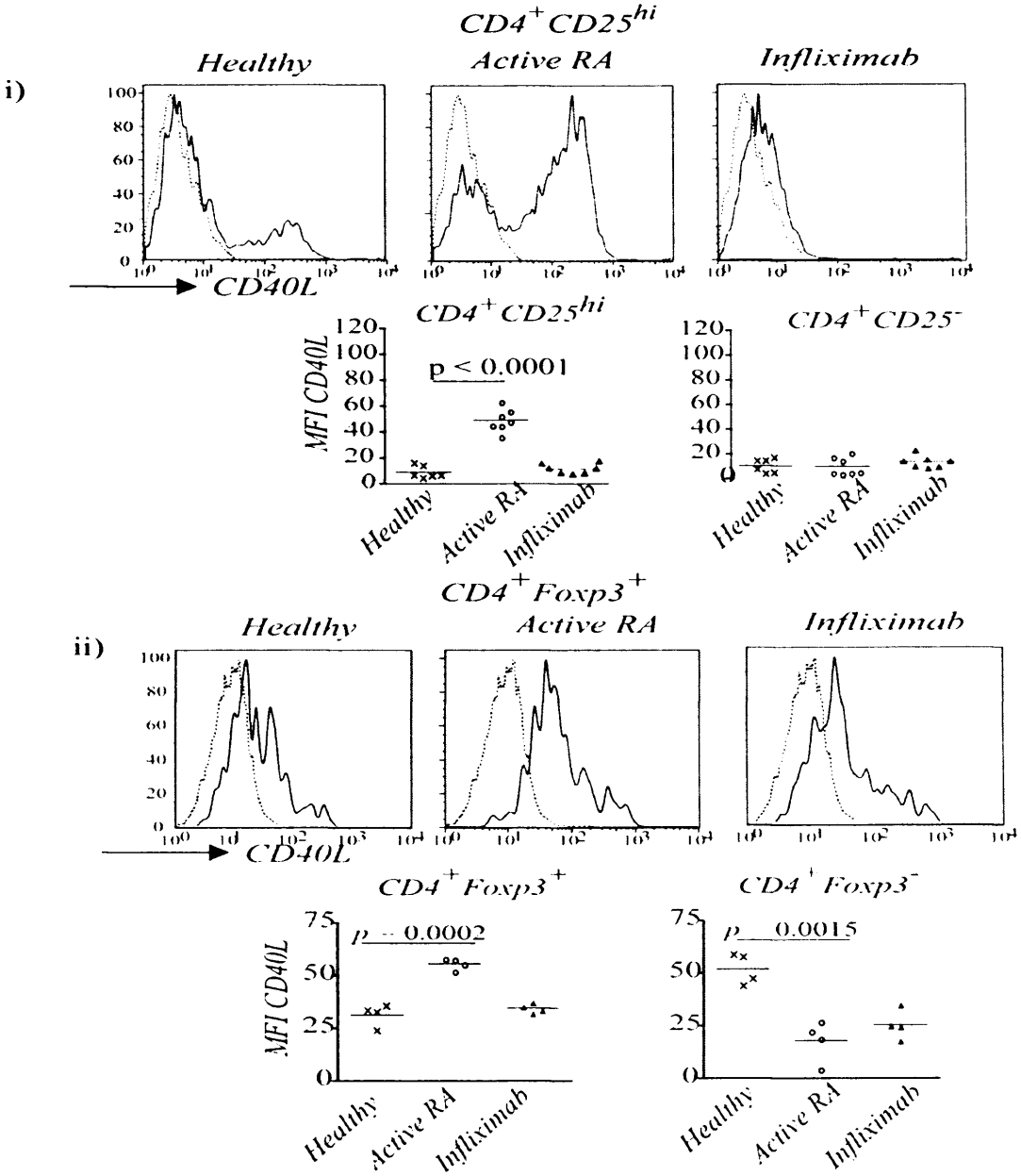


FIGURE 3.1.3.1 CD40L EXPRESSION IS INCREASED ON TREGS FROM ACTIVE RA PATIENTS

PBMC from healthy controls, active RA and infliximab patients were cultured for 4 hours with 50ng/ml PMA & 250ng/ml ionomycin. Following culture, cells were surface stained for CD4, CD25 and CD40L. Additionally, cells were intracellularly stained for Foxp3. Cells were gated on $CD25^{hi/-}$ or $Foxp3^{+/-}$ within the total $CD4^+$ T cell population (see figure 2.5 in materials & methods for gating strategies) Representative histogram plots shown; dotted lines illustrate isotype controls.

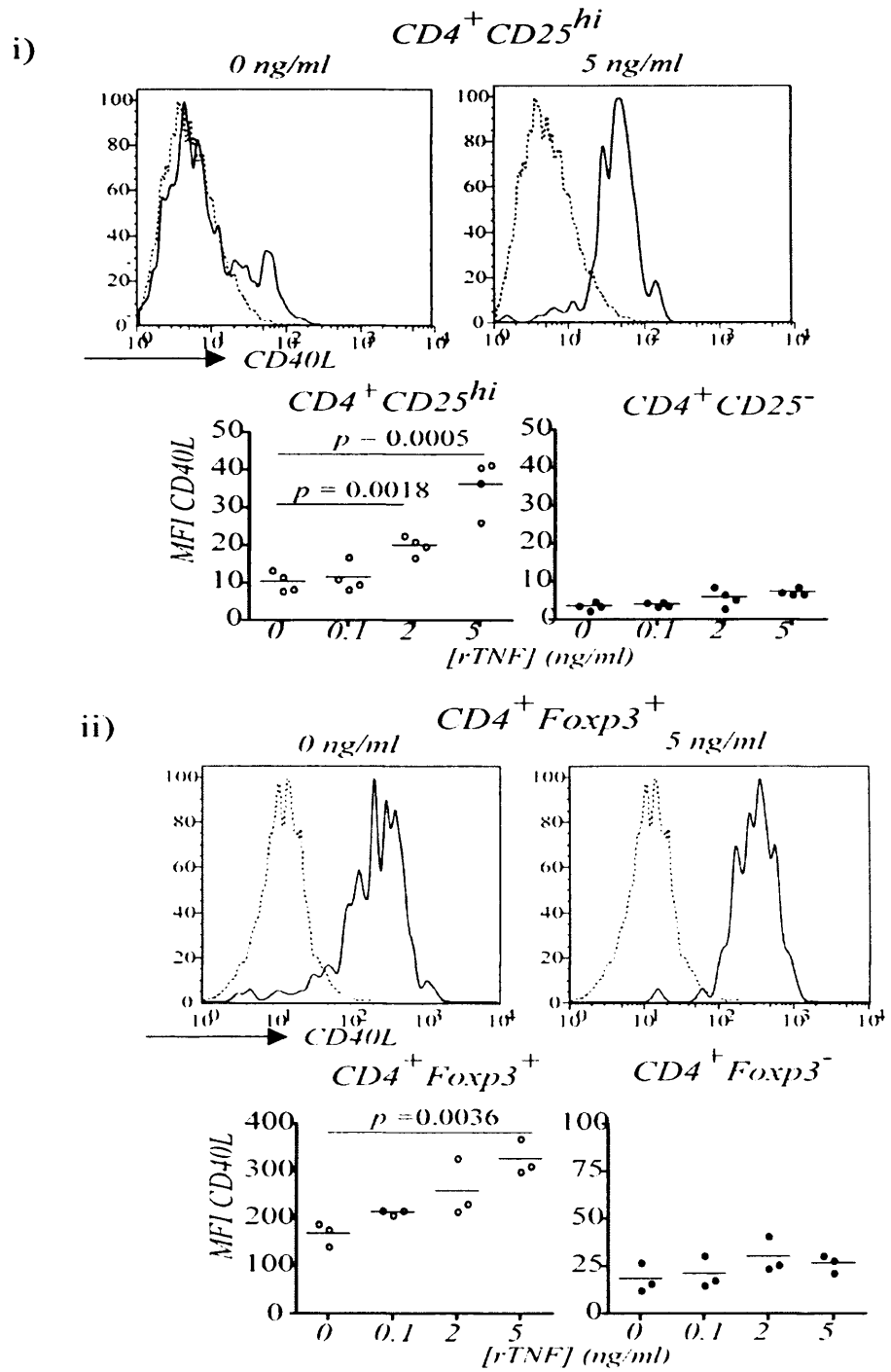


FIGURE 3.1.3.2 CD40L IS UP REGULATED ON HEALTHY TREGS IN THE PRESENCE OF TNF-α.

PBMC from healthy controls were cultured for 24 hours with 0.75μg/ml plate-bound anti-CD3 in the presence of increasing concentrations of recombinant TNF-α (0, 0.1,2 and 5ng/ml). Following culture, cells were stained and analysed as in 3.13.1.

CD40L expression on $CD4^+Foxp3^+$ Tregs increased in a dose-dependent fashion with increasing TNF-α concentration. However, there was a 10-fold increase in CD40L MFI on $Foxp3^+$ Tregs, compared $CD4^+CD25^{hi}$ Tregs (for example, mean MFI 350 and 35, respectively at 5ng/ml) (figure 3.1.3.1 ii). As with CCR5, this increase in MFI suggests that CD40L expression is much greater per cell on $CD4^+Foxp3^+$ Tregs. No significant difference in CD40L expression was observed $CD4^+Foxp3^-$ T effectors.

The observation that CD40L expression did not change in healthy $CD4^+Foxp3^-$ T effectors in the presence of TNF-α is interesting. This is because *ex vivo* analysis on healthy $CD4^+Foxp3^-$ T effectors demonstrated significantly high levels of CD40L expression (figure 3.1.3.1 ii), with a mean MFI of 50. This was reduced by half following plate-bound anti-CD3 stimulation in the absence of recombinant TNF-α (mean MFI 25, figure 3.1.3.2 ii), which was not observed on $CD4^+CD25^-$ T effectors. This is in contrast to the effects seen following plate-bound anti-CD3 stimulation on healthy Tregs, suggesting that stimulation via CD3 has differential effects on $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ T cells in terms of CD40L expression.

3.1.4 CTLA-4 EXPRESSION VARIES ON $CD4^+CD25^{hi}$ AND $CD4^+FOXP3^+$ TREGS FROM ACTIVE RA AND INFLIXIMAB PATIENTS EX VIVO, AND ON HEALTHY TREGS IN THE PRESENCE OF TNF-α

The analyses of CCR5 and CD40L have shown differences in expression levels on Tregs from active RA patients compared with Tregs from healthy controls and infliximab treated patients, indicating a migratory and co-stimulatory defect in these suppressor cells from active RA patients. CTLA-4 (CD152) is constitutively expressed on Tregs that serves to dampen immune responses by binding to its ligands the B7 molecules expressed on the surface of APC. Due to the constitutive expression on Tregs and immunoregulatory role CTLA-4 has, it was hypothesised that Tregs from active RA patients might be defective due to their inability to express high levels of CTLA-4.

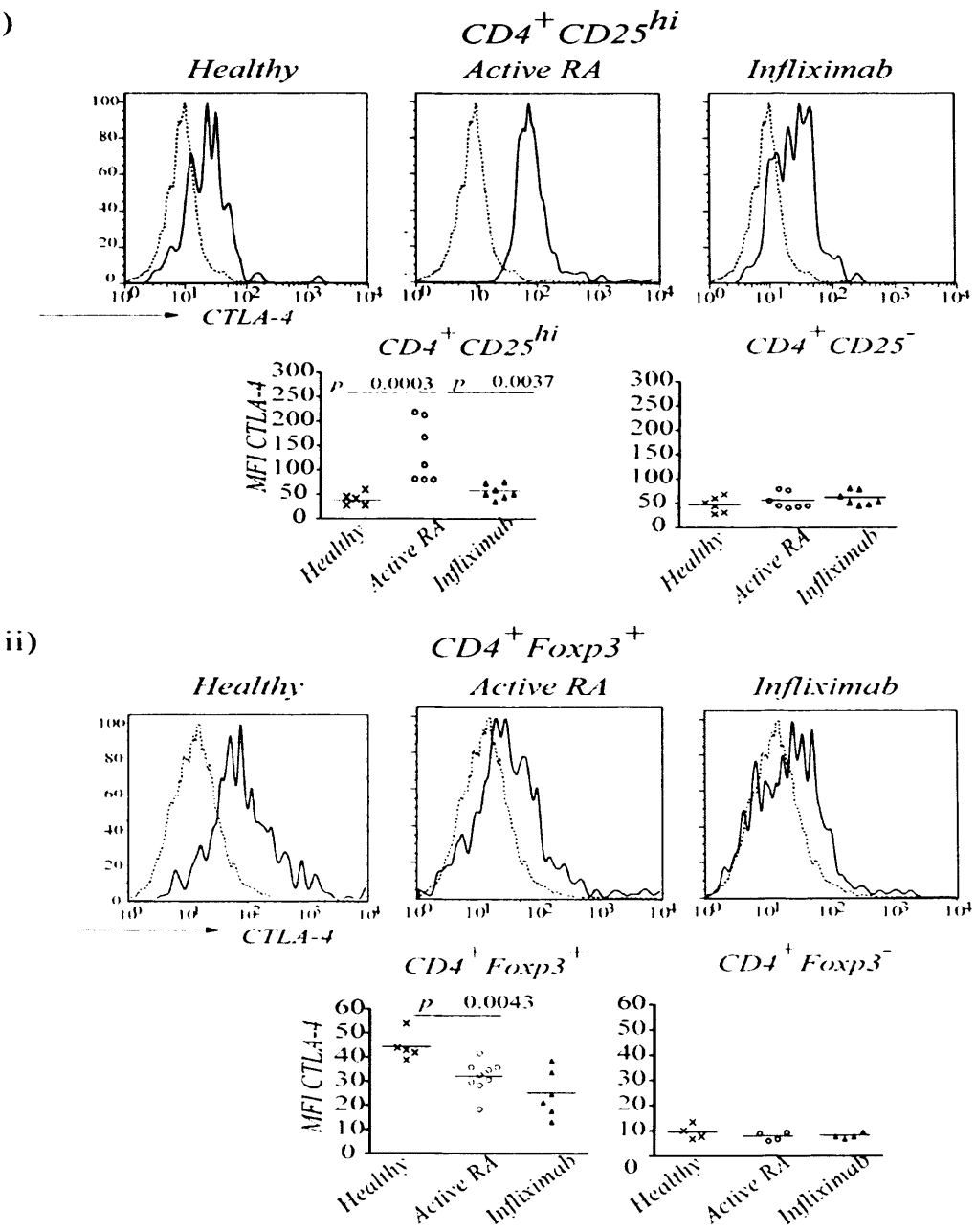


FIGURE 3.1.4.1 CTLA-4 EXPRESSION VARIES ON CD4$^+$CD25^{hi} AND CD4$^+$FOXP3$^+$ TREGS IN ACTIVE RA AND INFLIXIMAB PATIENTS

PBMC from healthy controls, active RA and infliximab patients were surface stained for CD4 and CD25, and intracellularly stained for CTLA-4. Additionally, cells were intracellularly stained for Foxp3. Cells were gated on CD4$^+$CD25$^{hi/-}$ or CD4$^+$Foxp3$^{+/-}$ within the total CD4$^+$ population. (See figure 2.5 in materials and methods for gating strategies). Representative histogram plots shown; dotted lines illustrate isotype controls.

PBMC from healthy controls, active RA an infliximab patients were surface stained for CD4, CD25 and intracellularly stained for CTLA-4. A highly significant increase in the expression of CTLA-4 on $CD4^+CD25^{hi}$ Tregs from active RA patients was observed, when compared to the same T cell population from healthy controls ($p = 0.0003$; see figure 3.1.4.1 i).

Patients with active disease had a 5-fold increase in expression of CTLA-4, with a mean MFI of 150, in contrast to a mean MFI of just 45 on $CD4^+CD25^{hi}$ Tregs from healthy controls. Interestingly, there was a 3-fold reduction in CTLA-4 expression on $CD4^+CD25^{hi}$ Tregs from infliximab-treated RA patients when compared to active RA patients ($p = 0.0037$). No difference was observed in CTLA-4 MFI on $CD4^+CD25^{hi}$ Tregs between healthy controls and infliximab patients. As with CCR5 and CD40L expression, the difference in phenotype between healthy controls and active RA patients did not continue in the T effector population, with respect to CTLA-4. In all three patient groups, expression was low, with no statistical significance. Levels of expression were similar to that seen on healthy $CD4^+CD25^{hi}$ Tregs. The observed expression of CTLA-4 on $CD4^+CD25^{hi}$ Tregs from active RA patients was surprising, given the hypothesis that active RA Tregs might be defective due to low levels of CTLA-4 expression. It was therefore necessary to compare CTLA-4 expression between $CD4^+CD25^{hi}$ and $CD4^+Foxp3^+$ Tregs.

PBMC from healthy controls, active RA and infliximab patients were surface stained for CD4, and intracellularly stained for Foxp3 and CTLA-4. Analysis of CTLA-4 expression on $CD4^+Foxp3^+$ Tregs revealed contrasting results to that observed with $CD4^+CD25^{hi}$ Tregs. MFI of CTLA-4 remained the same on $CD4^+Foxp3^+$ as on $CD4^+CD25^{hi}$ Tregs from healthy controls (compare figure 3.1.4.1 i and ii), with MFIs averaging to 45 in both populations of Tregs. However, analysis of CTLA-4 expression in active RA patients revealed that there was a significant down-regulation on $CD4^+Foxp3^+$ Tregs ($p = 0.0043$; figure 3.1.4.1 ii), when compared to healthy controls and $CD4^+CD25^{hi}$ Tregs from the same patients.

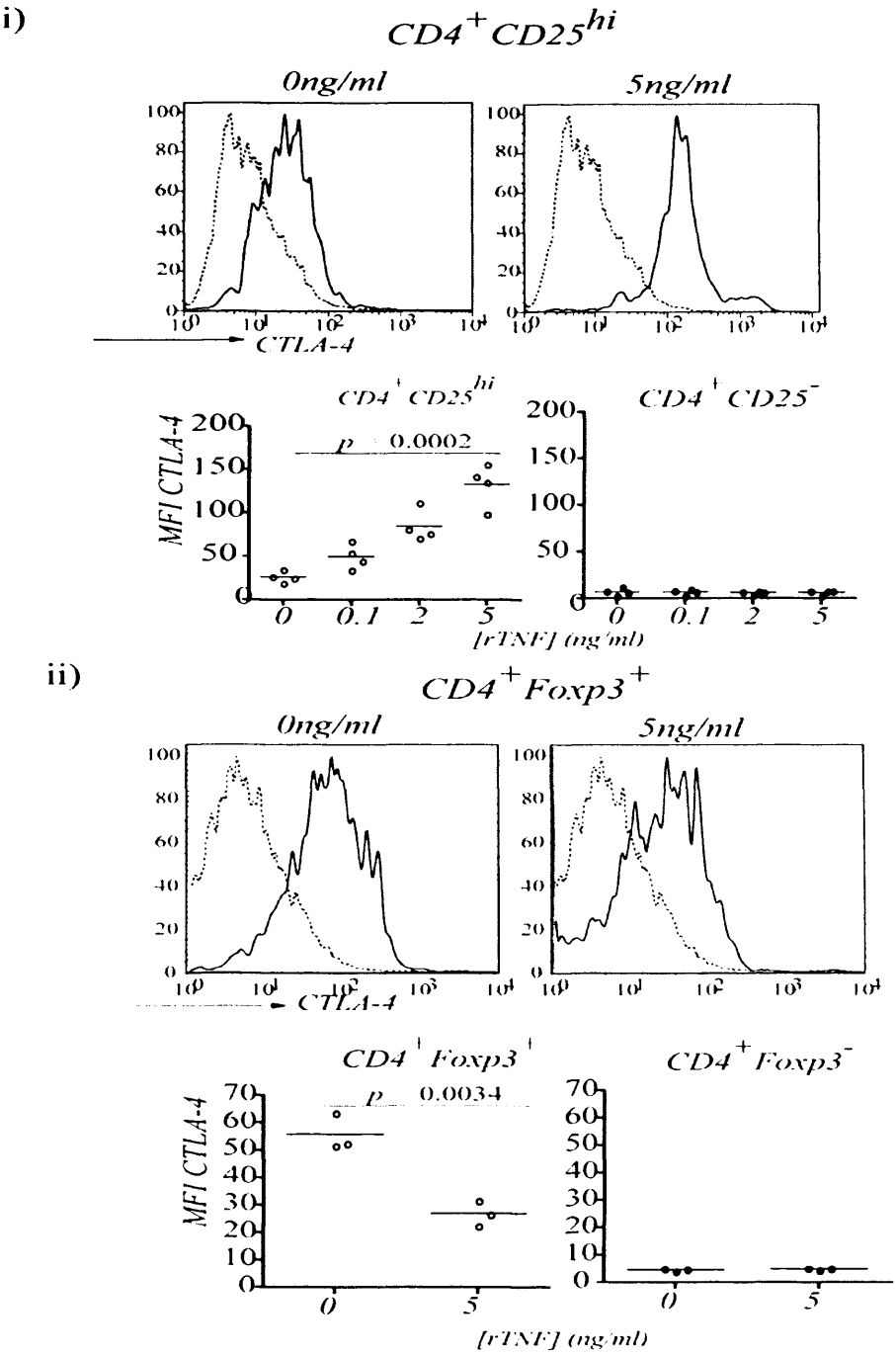


FIGURE 3.1.4.2 CTLA-4 EXPRESSION VARIES ON HEALTHY TREGS IN THE PRESENCE OF TNF-α.

PBMC from healthy controls were cultured for 24 hours with 0.75μg/ml plate-bound anti-CD3 in the presence of increasing recombinant TNF-α concentrations (0, 0.1, 2 and 5ng/ml). Following culture, cells were stained and analysed as in 3.1.4.1

The addition of recombinant TNF-α to healthy Tregs revealed a similar CTLA-4 expression pattern between $CD4^+CD25^{hi}$ and $CD4^+Foxp3^+$ Tregs, in the presence of increasing TNF-α concentration. There was a highly significant (p = 0.0002) dose-dependent increase in CTLA-4 expression on $CD4^+CD25^{hi}$ Tregs in the presence of TNF-α - CTLA-4 MFI increased from 30 in the absence of TNF-α to 150 in the presence of 5ng/ml (figure 3.1.4.2 i). These CTLA-4 MFI values were comparable to MFI values seen on healthy and active RA $CD4^+CD25^{hi}$ Tregs *ex vivo*.

3.1 DISCUSSION

3.1.1 TNF-α DOES NOT AFFECT FOXP3 EXPRESSION IN VIVO OR IN VITRO, BUT IN VIVO BLOCKADE UP-REGULATES EXPRESSION

Analysis of Foxp3 expression in active RA patients revealed that there was no statistical difference in the percentage of these cells when compared to healthy controls (figure 3.1.1.1). This result was mirrored in healthy Tregs that were exposed to increasing concentration of TNF-α (figure 3.1.1.2). The finding that no difference in $CD4^+Foxp3^+$ number was observed in active RA patients compared to control was in agreement with previous findings from this lab, where it had been shown that there was no difference in the number of $CD4^+CD25^{hi}$ Treg population (255). These results suggest that the defect in Tregs seen in active RA patients is not due to a reduced number of the suppressor cells, and these results are similar to findings in other autoimmune diseases including type 1 diabetes (258) and multiple sclerosis (259), where defects in Treg suppression have also been observed.

The unaffected Foxp3 expression observed in healthy Tregs in the presence of TNF-α (figure 3.1.4 B) is in contrast to findings by other groups who have shown that the defect in RA Tregs, more specifically down-modulation of Foxp3, is attributed to TNF-α (260). Although the authors of this paper argue the low concentrations of recombinant TNF-α (i.e. between 5 and 20ng/ml) have minimal effects on the suppressive function and Foxp3 expression of

Tregs, they do provide evidence that at much higher TNF-α concentrations (50ng/ml), Treg suppression and Foxp3 are decreased. However, the range of recombinant TNF-α concentrations used in this work was based on physiological serum TNF-α levels found in active RA patients, where serum concentrations typically range between 0.2-5ng/ml (261). Therefore, any effect on Treg suppression and Foxp3 expression seen due to TNF-α concentrations above this range may not be physiologically representative.

Of particular interest was the observation that infliximab patients have significantly higher levels of Foxp3 expression compared to healthy controls and active RA patients. Although etanercept is also an anti-TNF-α drug, no statistical difference in the level of Foxp3 expression was detected, suggesting that the way the two drugs function is a determining factor in Foxp3 expression. A recent paper supports this hypothesis, where it has been demonstrated that RA patients treated with adalimumab have increased numbers of Tregs (262).Both infliximab and adalimumab function by binding to membrane TNF-α, whereas etanercept binds to soluble TNF-α

The combined observations that Foxp3 expression is not altered in active RA patients or in healthy Tregs in the presence of recombinant TNF-α, suggest TNF-α does not affect Foxp3 expression in already established Treg population present within an individual. However, the increase in Foxp3 expression seen in infliximab patients does suggest that TNF-α prevents the induction of a new population of $Foxp3^+$ T cells

3.1.2 CCR5 EXPRESSION IS DOWN REGULATED ON TREGS FROM ACTIVE RA PATIENTS AND ON HEALTHY TREGS IN THE PRESENCE OF TNF-α

The role of CCR5 in RA is to recruit leukocytes to the site of inflammation, i.e. the joint. This has major implications in the pathology of RA, since excessive leukocyte recruitment correlates with disease progression. Numerous studies looking at CCR5 expression on leukocytes have shown that there is an accumulation of $CCR5^+$ T cells within the synovium of patients with RA (263-265). Moreover, studies looking at Tregs in RA patients have shown that there is also an accumulation of functionally suppressive Tregs within the joint (266).

In spite of these findings, the results presented in 3.1.2 demonstrate that peripheral blood Tregs from active RA patients have significantly reduced levels of CCR5 expressed on their surface. These results suggest that migratory problems exist in Tregs from patients with active disease. A recent paper has demonstrated that CCR5 expression is vital for the preferential recruitment of $Foxp3^+$ T cells to sites of intestinal inflammation, and subsequent inhibition of disease. Furthermore, the paper also showed that blockade of CCR5 prevented $Foxp3^+$ Tregs migrating to the inflamed sites (267).

These findings support the hypothesis that Tregs from the peripheral blood of active RA patients are unable to migrate to the sites of inflamed joints and inhibit inflammation due to a down-regulation of CCR5 expression. Results from the paper mentioned above (266) demonstrate that Tregs are present within the joints of patients with RA, suggesting that Tregs are able to efficiently migrate to the joints – which would go against the current hypothesis. However, the authors discuss that these Tregs are being induced locally within the joint.

Analysis of CCR5 expression on Tregs from RA patients treated with infliximab show that the chemokine receptor is up regulated to levels similar to healthy controls (figure 3.1.2.1). This suggests that inhibition of TNF-α is responsible for restoring CCR5 levels, thereby allowing Tregs to migrate to the joint and suppress inflammation. These results also suggest that TNF-α is responsible for the CCR5 defect seen on Tregs from active RA patients.

CCR5 acts as a co-receptor for HIV entry into CD4+ T cells and macrophages. Studies looking at CCR5 expression in healthy human peripheral blood lymphocytes have shown that the addition of TNF-α results in a down modulation of CCR5 expression. Additionally, TNF-α binding to its receptor TNFR2 (but nor TNFR1) activates the transcription factor nuclear factor-kappa B (NF-κB). The consequence of this is the induction of CCR5-specific chemokines RANTES (regulated upon activation normal T cell secreted and expressed; also known as CCL5), MIP-1α (also known as CCL3) and MIP-1β (also known as CCL4). Subsequent binding to CCR5 leads to rapid receptor internalisation, thereby reducing surface expression of CCR5 (268). Moreover studies looking at chemokine expression in RA patients have shown that these chemokines are indeed increased (269). Taken together, this suggests a potential mechanism for the decreased surface expression of CCR5 seen on active RA Tregs and on healthy Tregs in the presence of TNF-α.

3.1.3 CD40L EXPRESSION IS UP REGULATED ON ACTIVE RA TREGS AND ON HEALTHY TREGS IN THE PRESENCE OF TNF-α

In contrast to CCR5 expression, CD40L expression was up regulated on active RA Tregs in comparison to healthy controls and infliximab treated patients, and this up-regulation was mirrored on healthy Tregs in the presence of increasing TNF-α. This was surprising, since CD40L expression is not normally seen on naïve Tregs, but is up regulated upon activation. Moreover, CD40L up-regulation has been observed on $CD4^+$ T cells from patients with RA (270) Given the role CD40L plays in co-stimulation of T cells, the results presented here may suggest that Tregs from active RA Tregs may be defective due to increased expression of CD40L on their surface.

The expression of CD40L on Tregs has been investigated in the maintenance of transplant tolerance. These studies have shown that the presence of CD40L on Tregs abrogates Treg-mediated graft survival and this is reversed upon blockade of CD40L (63). Moreover, blockade of CD40-CD40L promotes the induction of functionally suppressive Tregs, that are capable of prolonging graft survival (271, 272).

The observed increased levels of CD40L on active RA Tregs also suggest that active RA Treg interactions with DCs promote DC maturation. The maturation status of DCs is important in normal Treg homeostasis: immature DC are capable of inducing peripheral tolerance either by inducing (Tr1/Th3) and expanding (thymus-derived $CD4^+CD25^{hi}Foxp3^+$) regulatory T cell populations in the absence of "signal 2" which in turn promotes DC migration to the sites of inflammation. However, this is dependent on the absence of CD40-CD40L interactions (271). The altered CD40L phenotype on active RA Tregs therefore, may indicate that patients with RA also have an altered DC maturation state, where they have an increased mature: immature DC ratio.

Binding of CD40 to CD40L on activated T cells induces the production of TNF-α and IL-6 (273). In addition, over-expression of CD40L on T cells from the gut leads to the induction of IL-12 and TNF-α. These findings might help to explain why Tregs from RA patients are unable to suppress pro-inflammatory cytokines, since CD40-CD40L potentiate TNF-α, as well as IL-12 (and hence subsequent IFN-γ) production.

The link between TNF-α and CD40-CD40L appears to behave as a positive feedback loop, where TNF-α is induced by such interactions, and in turn, TNF-α up-regulates both CD40 and CD40L expression. This could explain the up-regulation of the ligand on active RA Tregs and increase in CD40L expression on healthy Tregs in the presence of increasing TNF-α concentration.

Interestingly, treatment of Crohn's with infliximab down-regulates CD40L expression, and subsequent CD40L-induced TNF-α production (274). This supports the observation that Tregs from infliximab patients express CD40L at levels similar to healthy controls (figure 3.1.3.1 and 3.1.3.2). The authors of this paper also demonstrate that infliximab down-regulates both CD40 on the APC and CD40L on T cell, and it would therefore be interesting to see if this is also the case with RA-treated infliximab patients, and given the association between CD40-CD40L interactions and DC maturation, it would be intriguing to investigate the difference between DC maturation in active RA and infliximab-treated patients. It could be hypothesised that infliximab patients have a higher immature : mature DC phenotype, that can promote the induction of functionally suppressive Tregs in the periphery.

Thus, the increased levels of CD40L on active RA on regulatory T cells may act as a positive feedback mechanism, where it could promote DC activation/maturation and provides an environment that supports and enhances the inhibition of Treg suppressor function.

3.1.4 CTLA-4 IS DIFFERENTIALLY EXPRESSED ON $CD4^+CD25^{HI}$ AND $CD4^+FOXP3^+$ TREGS IN ACTIVE RA PATIENTS

Analysis of CTLA-4 expression in $CD4^+CD25^{hi}$ and $CD4^+Foxp3^+$ Tregs revealed that CTLA-4 is differentially expressed on both populations of Tregs in active RA patients. While CTLA-4 expression was significantly up regulated on $CD4^+CD25^{hi}$ Tregs, it was significantly down regulated on $CD4^+Foxp3^+$ Tregs (figure 3.1.4.1). Analysis of CTLA-4 on healthy Tregs in the presence of TNF-α also revealed that CTLA-4 is differentially expressed between $CD4^+CD25^{hi}$ and $CD4^+Foxp3^+$ Tregs (figure 3.1.4.2).

The phenotypic difference in CTLA-4 expression between both population of Tregs in active RA patients suggest that $CD4^+CD25^{hi}$ Tregs are "activated" Tregs. It is tempting to simply call these cells activated T cells; however, previous work carried out in this lab and other labs has shown that $CD4^+CD25^{hi}$

Tregs from active RA patients are anergic *in vitro* (255, 266, 260) – a characteristic feature of Tregs. This suggests that there may be two subsets of Tregs within active RA patients, both of which retain some Treg features i.e. are anergic and/or express Foxp3, but are also defective. While the $CD4^+CD25^{hi}$ Tregs are defective due to their activated phenotype (i.e. increased levels of CD40L, CTLA-4), $CD4^+Foxp3^+$ Tregs are defective not only because they express increased levels of CD40L, but also because they have significantly low levels of CTLA-4 on their surface, thereby preventing them to suppress via cell contact.

Analysis of CTLA-4 is conventionally done intracellularly, since surface expression of the ligand is transient. The decrease in intracellular CTLA-4 suggests two potential hypotheses: Either, that active RA Tregs may have defective signalling mechanisms, which abrogates surface expression, The second hypothesis may be that there is augmented CTLA-4 recycling between the intracellular stores and the plasma membrane.

If the first hypothesis is true, then this would suggest aberrant association between CTLA-4 and the adaptor protein complex AP-1/AP-2, which is regulates transport of CTLA-4 to the surface. A specific motif (YVKKM) in the cytoplasmic tail of CTLA-4 binds to AP-2. If the tyrosine residue in this motif is phosphorylated, then the interaction between CTLA-4 and AP-2 is inhibited, and consequently, CTLA-4 remains on the cell surface (32). Phosphatases such as Src-homology (SH)-2 domain phosphates (SHP-2) have also been associated with CTLA-4 signalling (275). This signalling mechanism might explain the low levels of CTLA-4 in active RA Foxp3$^+$ Tregs: active RA Foxp3$^+$ Tregs might be associated with increased levels of phosphatases such as SHP-2, thereby limiting surface expression of CTLA-4.

The second hypothesis would suggest that intracellular stores of CTLA-4 are down regulated, due to increased recycling between intracellular stores and the plasma membrane. A recent paper looking at CTLA-4 trafficking in Tregs has shown that activation of the small GTPase ADP ribosylation factor (ARF)-1 and phospholipase D (PLD) - both of which are responsible for vesicle formation – facilitate the transport of CTLA-4 to the plasma membrane, which is accompanied with continued endocytosis (31). PLD activation is up regulated in the presence of TNF-α (276). This could suggest that the rate of CTLA-4 recycling in active RA Foxp3$^+$ Tregs is increased. Increased activation of PLD, via TNF-α would promote CTLA-4 recycling at a much greater rate than in normal control Foxp3$^+$ Tregs.

However, the second hypothesis would not explain the low levels of CTLA-4 expression in Tregs from infliximab patients (since TNF-α is inhibited). This observation was surprising, since Tregs from infliximab patients are functionally suppressive (255), suggesting that surface molecules, including CTLA-4, are returned to normal levels. The levels of CTLA-4 in infliximab patients were comparable to active RA Tregs (figure 3.1.4.1). Given that CTLA-4 is constitutively expressed on conventional Tregs, the observation that CTLA-4 remains low infliximab patients suggests that the anti-TNF-α drug is not simply restoring the Treg defect seen in active RA Tregs.

Instead, infliximab may be inducing a distinct population of Foxp3-expressing Tregs that do not require CTLA-4 to function, but instead function, for example, via TGF-β and/or IL-10.

CHAPTER 3.2

A DISTINCT POPULATION OF REGULATORY T CELLS IS INDUCED IN RA PATIENTS TREATED WITH INFLIXIMAB

3.2 RESULTS

Analysis of the various molecules mentioned in chapter 3.1 revealed interesting phenotypic differences in Tregs from patients with active RA, compared to healthy controls and RA patients treated with infliximab. However, of considerable interest were the observations of increased $Foxp3^{+}$ Treg numbers (Figure 3.1.4) and altered CD62L expression in infliximab-treated RA patients compared to healthy controls and active RA patients (see figure 3.2.1).

The combination of the above observations coupled with the presence of functionally suppressive Tregs in infliximab-treated RA patients suggested that neutralisation of TNF-α in RA patients does not necessarily restore Treg function. Instead, it may induce a new population of Tregs that differ both phenotypically and functionally when compared to conventional thymically derived Tregs from healthy controls and active RA patients. The following sections present a series of experiments that address the question of whether neutralisation of TNF-α in RA patients, via infliximab, induces a new population of Tregs that are not only phenotypically, but also functionally different to conventional Tregs.

3.2.1 INFLIXIMAB-TREATED RA PATIENTS HAVE AN INCREASED NUMBER OF CD62L$^-$ FOXP3$^+$ T CELLS

Foxp3 is a transcription factor that is associated with T cells that have regulatory function. In addition, Foxp3 is believed to be a more reliable marker for Tregs, since it is known that activated T cells also express, albeit transiently, CD25 on their surface. It has previously been shown that patients that have been treated with infliximab have an increased number of peripheral CD4+CD25^{hi} Tregs.

As it has already been demonstrated that that infliximab-treated RA patients have a significantly higher number of CD4$^+$Foxp3$^+$ T cells, it was necessary to determine whether these cells differed phenotypically, compared to Tregs from healthy controls and active RA patients. Co-staining of PBMC from all three study groups with CD62L and Foxp3 (in addition to CD4 and CD25 staining) revealed that healthy CD4$^+$Foxp3$^+$ T cells were predominantly CD62L$^+$, as were active RA CD4$^+$Foxp3$^+$ Tregs. However, CD4+Foxp3+ T cells from infliximab-treated patients were predominantly CD62L$^-$ (see figure 3.2.1 i). To better characterise the phenotype of Foxp3$^+$ Tregs from infliximab patients, cells were co-stained for CCR7 and CD45RO.

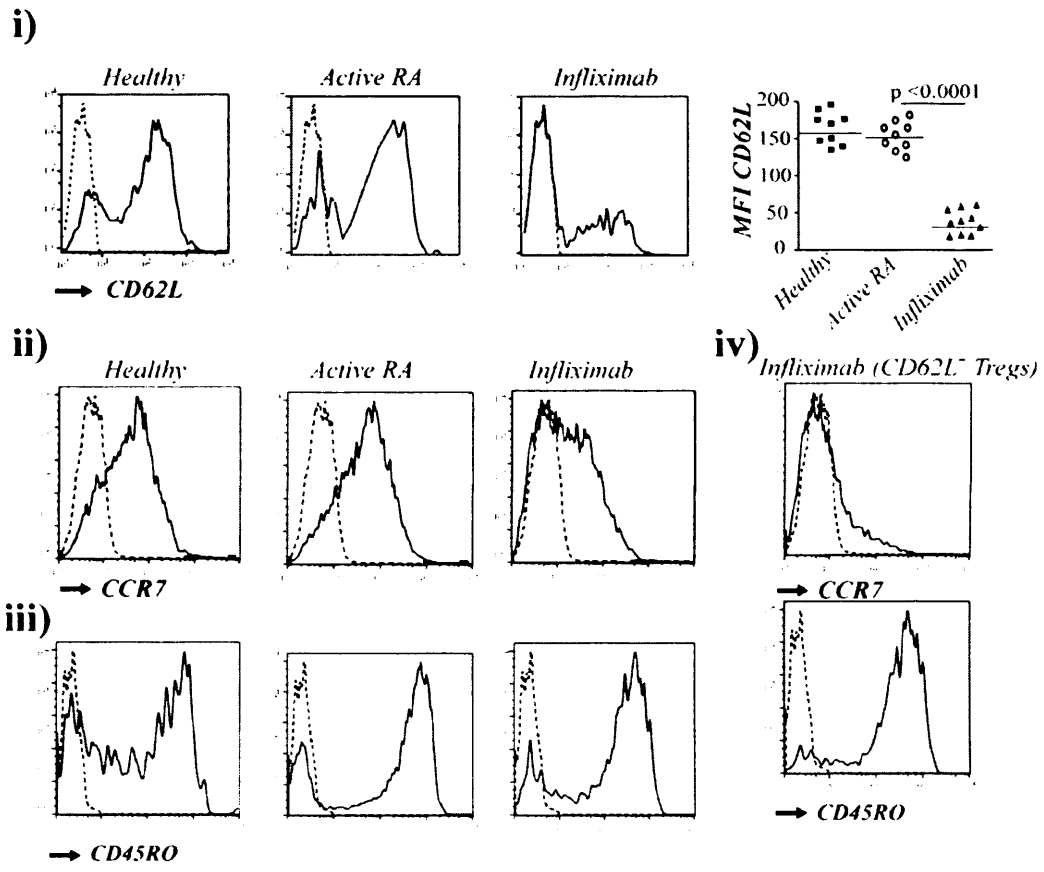


FIGURE 3.2.1 REGULATORY T CELLS FROM INFLIXIMAB-TREATED RA PATIENTS HAVE AN ALTERED CD62L PHENOTYPE COMPARED TO HEALTHY CONTROLS AND PATIENTS WITH ACTIVE DISEASE.

PBMC from healthy controls, active RA and infliximab-treated RA patients were surface stained for CD4 (APC), CD25 (PE-Cy5) and intracellularly stained for Foxp3 (Alexafluor-488). Additionally, cells were surface stained for CD62L (PE/PE-Cy7) i), CCR7 (PE-Cy7) (i, iv) and CD45RO (PE) (iii, iv). For analysis, cells were gated on $Foxp3^+$ cells, within the total $CD4^+$ T population (i, ii, iii), or within the $CD4^+CD62L^-Foxp3^+$ population (infliximab patients only; iv). Representative histogram plots shown; dotted lines illustrate isotype controls.

Analysis of $CD4^+Foxp3^+$ T cells in relation to these two surface markers revealed that CCR7 expression was significantly diminished in infliximab-treated patients, compared to healthy controls and active RA patients, whereas CD45RO expression was relatively similar in all three study groups (figure 3.2.1 ii and iii, respectively). Further analysis of CCR7 and CD45RO expression showed that $CD62L^-Foxp3^+$ Tregs from infliximab patients lack CCR7, but remain $CD45RO^+$ (figure 3.2.1 iv)

3.2.2 CD62L⁻ Tregs from infliximab-treated patients are more potent suppressors than their CD62L⁺ counterparts and suppress via TGF-β and IL-10

In addition to their characteristic phenotype, such as CD25 and Foxp3, Tregs are defined by their functional properties. Two main functions are characteristic of Tregs: Suppression of T effector proliferation and pro-inflammatory cytokines, namely TNF-α and IFN-γ. Although the above results suggest that Tregs from infliximab-treated patients differ phenotypically, in terms of CD62L and CCR7 expression, it was necessary to determine whether $CD62L^-$ Tregs from these patients are functionally suppressive.

In order to address this issue, PBMC from healthy controls, active RA and infliximab-treated RA patients were surface stained for CD4, CD25 and CD62L. The cells were then FACS-sorted into three distinct populations: $CD4^+CD25^-$ (referred to hereafter as T effectors), $CD4^+CD25^{hi}CD62L^+$ ($CD62L^+$ Tregs) and $CD4^+CD25^{hi}CD62L^-$ ($CD62L^-$ Tregs) (see figure 2.2) Following isolation, $CD62L^+$ or $CD62L^-$ Tregs were cultured with autologous T effectors: regulatory T cell ratios of 3:1, 2:1 and 1:1, for 5 days with 2μg/ml of soluble anti-CD3 and 2μg/ml soluble anti-CD28, followed by addition of thymidine in the last 18 hours of culture.

Figure 3.2.2.1 i shows the effect $CD62L^+$/$CD62L^-$ Tregs have on T effector proliferation when cultured at a 2:1 ratio. T effector proliferation was better suppressed in the presence of $CD62L^+$ Tregs from healthy controls - these Tregs were three times more suppressive than $CD62L^-$ Tregs, which is in agreement with previous published findings (106).

Analysis of the inhibition of T effector proliferation by active RA Tregs revealed that of the two sub-populations, $CD62L^+$ Tregs were marginally better at suppressing than $CD62L^-$ Tregs. However, $CD62L^+$ Tregs from active RA patients were approximately 20% less effective at suppressing than $CD62L^+$ Tregs from healthy controls (figure 3.2.2.1 i).

Analysis of regulatory T cell suppressor activity from infliximab-treated patients revealed that there was a reversal in the suppressive capabilities between $CD62L^+$ and $CD62L^-$ Tregs: $CD62L^-$ Tregs were found to be significantly more potent suppressors ($p<0.0001$) of T effector proliferation than their $CD62L^+$ counterparts (figure 3.2.2.1). Further comparisons of the suppressive capabilities of $CD62L^-$ Tregs from these patients show that these cells suppress at similar, if not the same, levels as $CD62L^+$ Tregs from healthy controls (figure 3.2.2.1).

Consistent with the results obtained from measuring T cell proliferation, $CD62L^+$ Tregs from healthy controls were better suppressors than $CD62L^-$ Tregs, and $CD62L^-$ Tregs from treated patients were better at suppressing both TNF-α and IFN-γ than $CD62L^+$ Tregs (see figure 3.2.2.1 ii). In agreement with previous findings, the ability of Tregs (both $CD62L^+$ and $CD62L^-$) from active RA patients to suppress both TNF-α and IFN-γ production was considerably reduced (figure 3.2.2.1 ii). Further analysis of $CD62L^+$ Tregs from infliximab-treated patients showed that these cells suppressed at levels similar to that observed by $CD62L^+$ Tregs from active RA patients figure 3.2.2.1 ii), suggesting that $CD62L^+$ Tregs are still defective in infliximab-treated patients. This finding raised the possibility that infliximab therapy was not simply restoring the functional defect seen in active RA patients.

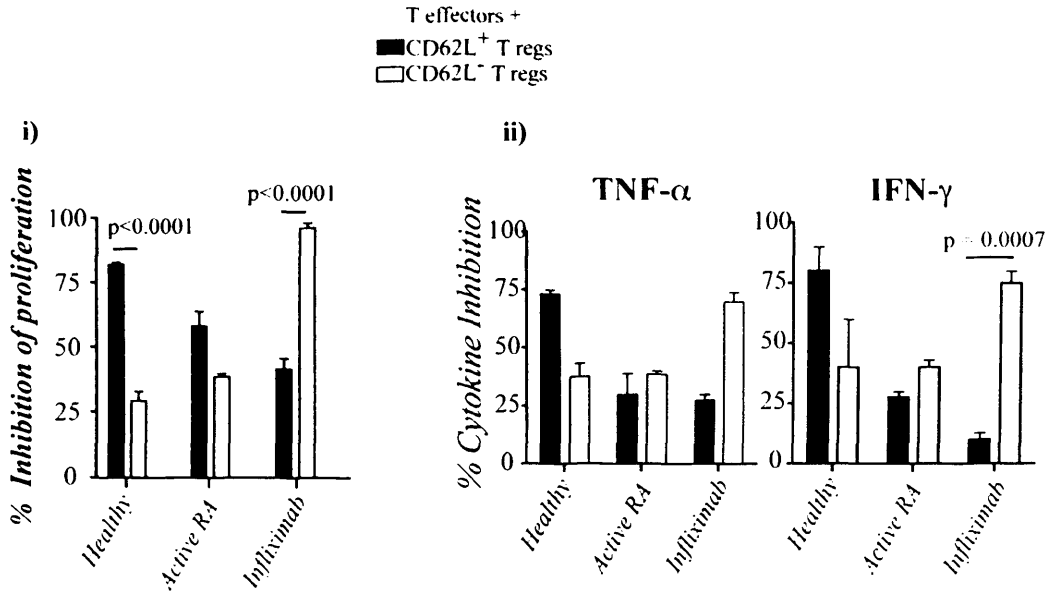


FIGURE 3.2.2.1 CD62L⁻ REGULATORY T CELLS FROM INFLIXIMAB-TREATED RA PATIENTS ARE MORE POTENT SUPPRESSORS THAN THEIR CD62L⁺ COUNTERPARTS.

PBMC were MoFlo sorted, as described in materials and methods. (i) T effectors were co-cultured with either $CD62L^+$ Tregs (black bars) or $CD62L^-$ Tregs (white bars) at Teff:Treg ratios 3:1, 2:1 and 1:1 (2:1 ratio shown). (ii) T effectors were cultured alone or co-cultured at a 2:1 ratio with either $CD62L^+$ or $CD62L^-$ T regs as described in materials and methods for intracellular staining of TNF-α and IFN-γ. Results are means of 3 experiments. Error bars represent SEM. See Appendix III for mean raw proliferation values.

The change in phenotype of Tregs from infliximab-treated patients suggested that these Tregs might not be thymically derived cells, but rather induced within the periphery. TGF-β and IL-10 are cytokines, which are implicated in the homeostasis and function of adaptive Tregs (Th3, Tr1 cells). This raised the question as to whether either or both of these cytokines are involved in the function of $CD62L^-$ Tregs from treated patients. In order to address this question, PBMC from healthy controls and infliximab-treated patients were FACS sorted into the $CD62L^+$ and $CD62L^-$ Tregs, as well as T effectors. T effectors were co-cultured at a 2:1 ratio with either $CD62L^+$ or $CD62L^-$ Tregs with 2μg/ml anti-CD3 and 2μg/ml soluble anti-CD28 in the presence or absence of neutralising antibody to TGF-β and/or neutralising antibody to IL-10.

Data in figure 3.2.2.2 show that suppression of TNF-α or IFN-γ by healthy $CD62L^+$ or $CD62L^-$ Tregs was not affected by the addition of anti-TGF-β, anti-IL-10 or both neutralising antibodies. In contrast, analysis of Tregs from infliximab-treated patients revealed that neutralisation of TGF-β significantly impaired the ability of $CD62L^-$ Tregs to suppress both TNF-α ($p < 0.01$) and IFN-γ ($p < 0.005$). Neutralisation of IL-10 in these cultures did affect the ability of CD62L- Tregs to suppress cytokine production, but did so less effectively than neutralisation of TGF-β. Blockade of IL-10 induced a 25% reduction in the ability of $CD62L^-$ Tregs to suppress, compared to a 50% reduction in the presence of anti-TGF (figure 3.2.2.2). Blockade of both TGF-β and IL-10 completely abrogated suppression. Interestingly, the addition of neutralising antibodies to cultures containing $CD62L^+$ Tregs from infliximab patients had opposing effects to cultures containing $CD62L^-$ Tregs. Blockade of TGF-β induced a reversal in suppressive capability of $CD62L^+$ Tregs, with a highly significant inhibition of both TNF-α and IFN-γ ($p=0.0002$) (figure 3.2.2.2). The addition of neutralising antibody to IL-10 alone, or in combination with anti-TGF-β also induced suppression via $CD62L^+$ Tregs, but was not as effective as neutralising TGF-β.

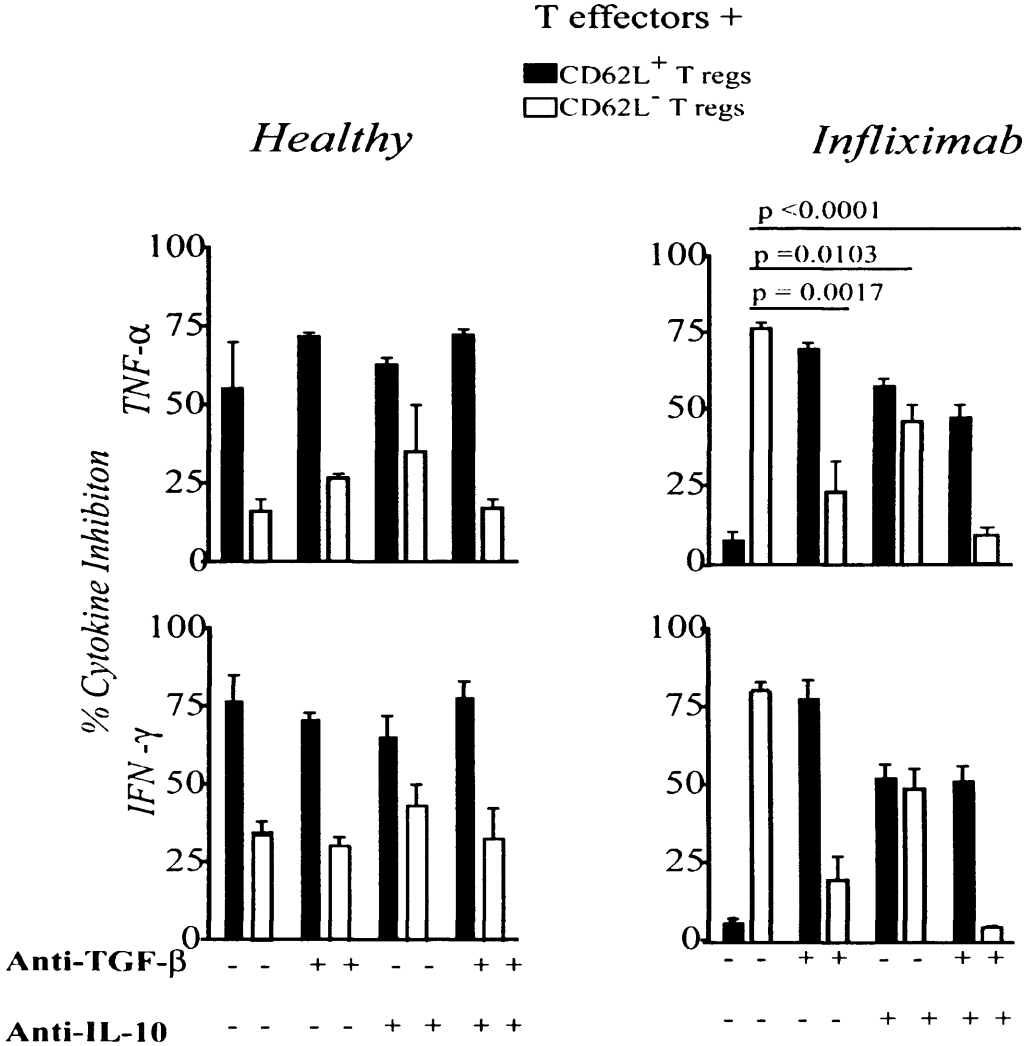


FIGURE 3.2.2.2 CD62L⁻ TREGS FROM INFLXIMAB PATIENTS SUPPRESS VIA TGF-β AND IL-10

PBMC were MoFlo sorted, as described in materials and methods. T effectors from healthy and infliximab-treated RA patients were cultured alone or co-cultured as in (3.2.2.1) for 48 hours with 2μg/ml anti-CD3 and anti-CD28, or in the presence of 2μg/ml anti-TGF-β1, 0.5μg/ml anti-IL-10, or both antibodies. Cells were intracellularly stained for TNF-α and IFN-γ. See Appendix III for mean raw cytokine values.

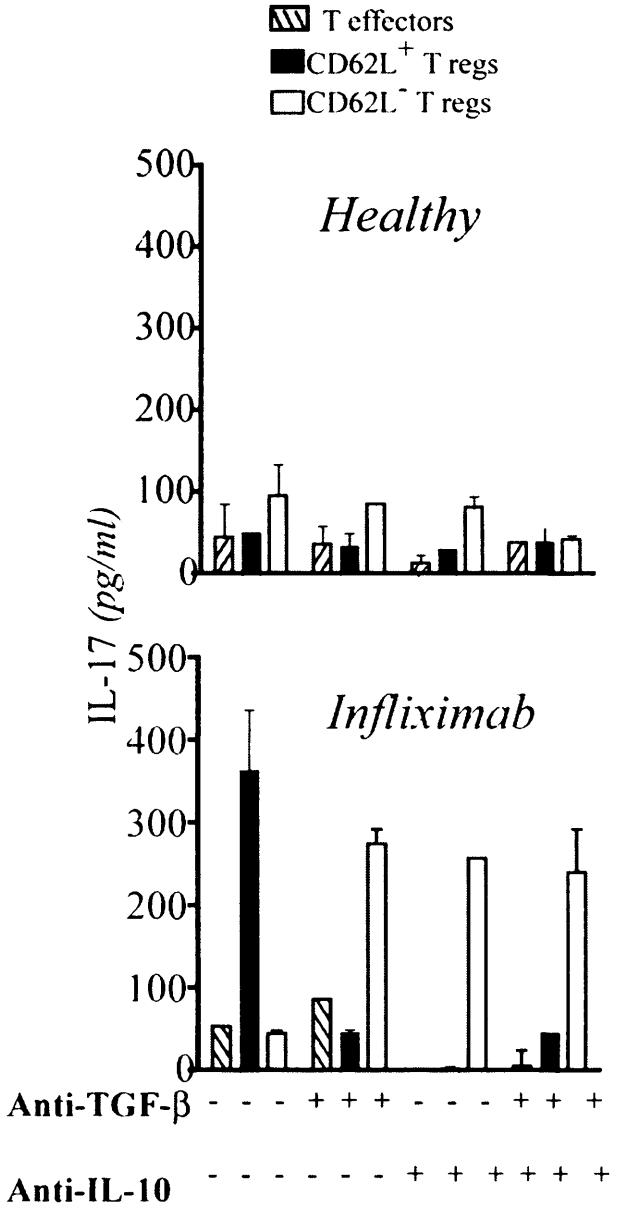


FIGURE 3.2.2.3 BLOCKADE OF TGF-β AND IL-10 HAS DIFFERENTIALL EFFECTS ON IL-17 PRODUCTION BETWEEN CD62L+ AND CD62L- TREGS FROM INFLXIMAB PATIENTS

T effectors (lined bars), $CD62L^+$ and $CD62L^-$ Tregs from healthy controls and infliximab patients were cultured as in (3.2.2.2) for 72 hours. Supernatants were collected and analysed for IL-17 by ELISA. Results are means of 3 experiments. See Appendix III for mean raw cytokine values.

The opposing effects of neutralising TGF-β and IL-10 on $CD62L^-$ and $CD62L^+$-mediated cytokine inhibition, suggested that blockade of TNF-α alone is not sufficient to reverse the defect in RA Tregs, and that these cytokines, particularly TGF-β, have differing effects on thymically-derived $CD62L^+$ and induced $CD62L^-$ Tregs in infliximab-treated RA patients. Given the increasing data and evidence surrounding the role of TGF-β in IL-17/Th17 induction, it was important to investigate whether IL-17 is produced by either T effectors, $CD62L^+$ or $CD62L^-$ Tregs from infliximab patients. Consequently, these subsets of cells from healthy controls and infliximab patients were cultured for 72 hours with anti-CD3/anti-CD28 in the presence or absence of neutralising antibody to TGF-β and/or anti-IL-10. Figure 3.2.2.3 shows that IL-17 production from the above subsets of cells of healthy controls produce extremely low levels of IL-17 in all culture conditions. T effectors from infliximab patients (lined bars) also produced low levels of IL-17, despite the presence of either neutralising antibody. Interestingly, $CD62L^+$ Tregs from infliximab patients produced high levels of IL-17 (approx 400 pg/ml), which was dramatically reduced in the presence of neutralising antibody to TGF-β and/or neutralising antibody to IL-10 (50pg/ml).

Once again, blockade of either TGF-β or IL-10 or both cytokines had differing effects on $CD62L^-$ Tregs from infliximab patients. Polyclonal stimulation alone resulted in very low levels of IL-17 (approx. 40pg/ml); however, blockade of TGF-β and/or IL-10 induced significant increases in the levels of IL-17 (approx 280 pg/ml), although these levels were lower than IL-17 levels produced by $CD62L^+$ Tregs following polyclonal stimulation.

3.2.3 CD62L$^-$ TREGS FROM INFLIXIMAB PATIENTS FUNCTION IN A PARTIAL CELL CONTACT-DEPENDENT FASHION & PROMOTE IL-10 PRODUCTION

Numerous studies have demonstrated that thymically derived Tregs function in a cell contact-dependent, cytokine-independent fashion, although emerging studies also indicate that thymically derived, as well as adaptive, Tregs rely on TGF-β for their peripheral maintenance and/or function. Moreover, previous published work from this lab has shown that $CD4^+CD25^{hi}$ Tregs from infliximab patients function in a cell contact-dependent fashion. However, one major difference between the original work and present study, is that Tregs in the present study have been divided into sub-populations, which may have differing modes of function, compared to $CD4^+CD25^{hi}$ Tregs in general. Moreover, results of the neutralisation experiments described above also indicate that $CD62L^-$ Tregs from infliximab-treated patients may function in a cell contact- independent fashion.

Results from the neutralisation assays, as well as previous work done in this lab, suggest that both cytokines and cell contact are implicated in the way $CD62L^-$ Tregs from infliximab patients function. Recent evidence also suggests that the cell contact-dependent nature of Treg function is mediated by membrane-bound TGF-β (131). Membrane-bound TGF-β can be analysed in its inactive form, bound to LAP. In order to determine how TGF-β is involved in the function of $CD62L^-$ Tregs from infliximab patients, Treg cells were cultured alone for five days, adapted from methods discussed in (131).

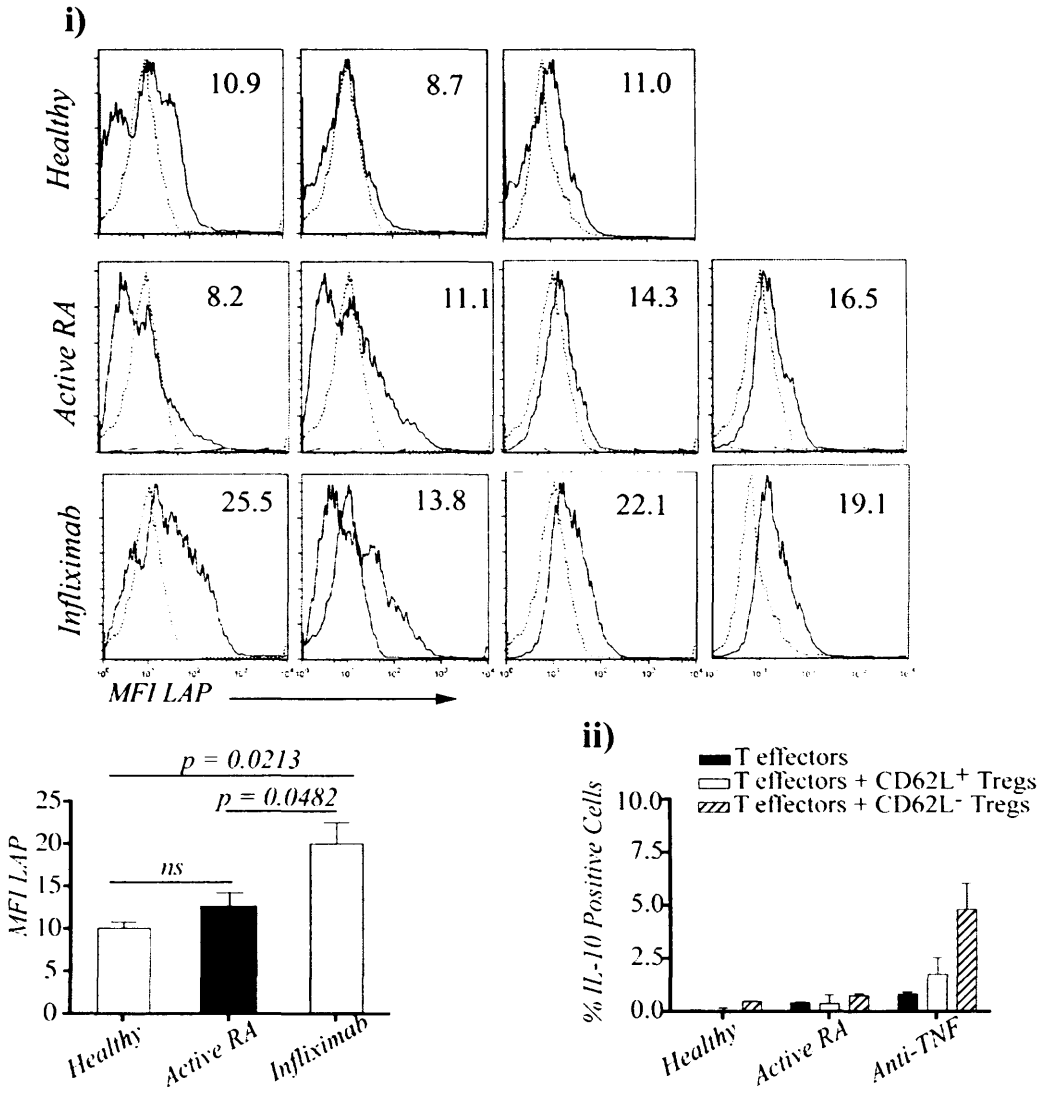


FIGURE 3.2.3 CD62L⁻ TREGS FROM INFLIXIMAB PATIENTS EXPRESS HIGH LEVELS OF TGF-β ON THEIR SURFACE AND PROMOTE IL-10 PRODUCTION

(i) Tregs from healthy controls and infliximab patients were isolated by MACS and stimulated with 2μg/ml of soluble anti-CD3 and anti-CD28, and FACS stained for LAP. Histogram plots shown are of three individual healthy controls and four infliximab patients. (ii) T effectors, $CD62L^+$ and $CD62L^-$ Tregs from healthy controls, active RA and infliximab patients were isolated via MoFlo. T effectors were either culture alone or co-cultured for 48 hours (PMA and ionomycin added in the last 5 hours of culture with $CD62L^+$ or $CD62L^-$ Tregs and intracellularly stained for IL-10.

Tregs from healthy controls, active RA and infliximab patients were subsequently stained for LAP. The histogram plots in figure 3.2.3 i show that Tregs from infliximab patients express significantly higher levels of LAP compared to healthy controls ($p = 0.0218$). Moreover, analysis of LAP on active RA Tregs shows that there was no significant difference in mean fluorescence intensities (MFI) when compared with healthy controls, but is significantly lower when compared to infliximab-treated patients ($p = 0.0482$).

Further investigation into how $CD62L^-$ Tregs from infliximab patients function has shown that these Tregs, when co-cultured with T effectors, promote the production of IL-10 (figure 3.2.3 ii). T effectors from healthy controls, active RA and infliximab patients were either cultured alone or co-cultured with FACS-sorted $CD62L^+$ and $CD62L^-$ Tregs for 48 hours with anti-CD3 and anti-CD28, and stimulated in the final 5 hours with PMA and ionomycin. Cells were then intracellularly stained for IL-10. Figure 3.2.3 ii shows that T effectors from healthy controls and active RA patients produce negligible amounts of IL-10 when cultured alone or in the presence of either subset of Tregs. By contrast, cultures containing T effectors and $CD62L^-$ Tregs from infliximab patients result in high numbers of cells that stain positively for IL10, which is not seen with T effectors cultured with $CD62L^+$ Tregs from the same patient group.

3.2.4.1 In vitro addition of infliximab to active RA T effectors induces a population of FoxP3$^-$ T cells

Experiments so far have shown that infliximab-treated patients have a distinct population of Tregs that differ phenotypically from healthy Tregs, in terms of CD62L expression. In order to determine whether these Tregs arise due to infliximab treatment itself, *in vitro* experiments using infliximab in cultures were carried out. Conventional thymically derived Tregs are $CD62L^+$. This suggested that Tregs from infliximab-treated patients are not derived from the natural population of suppressor cells, but are induced in the periphery from a non-Treg or T effector population.

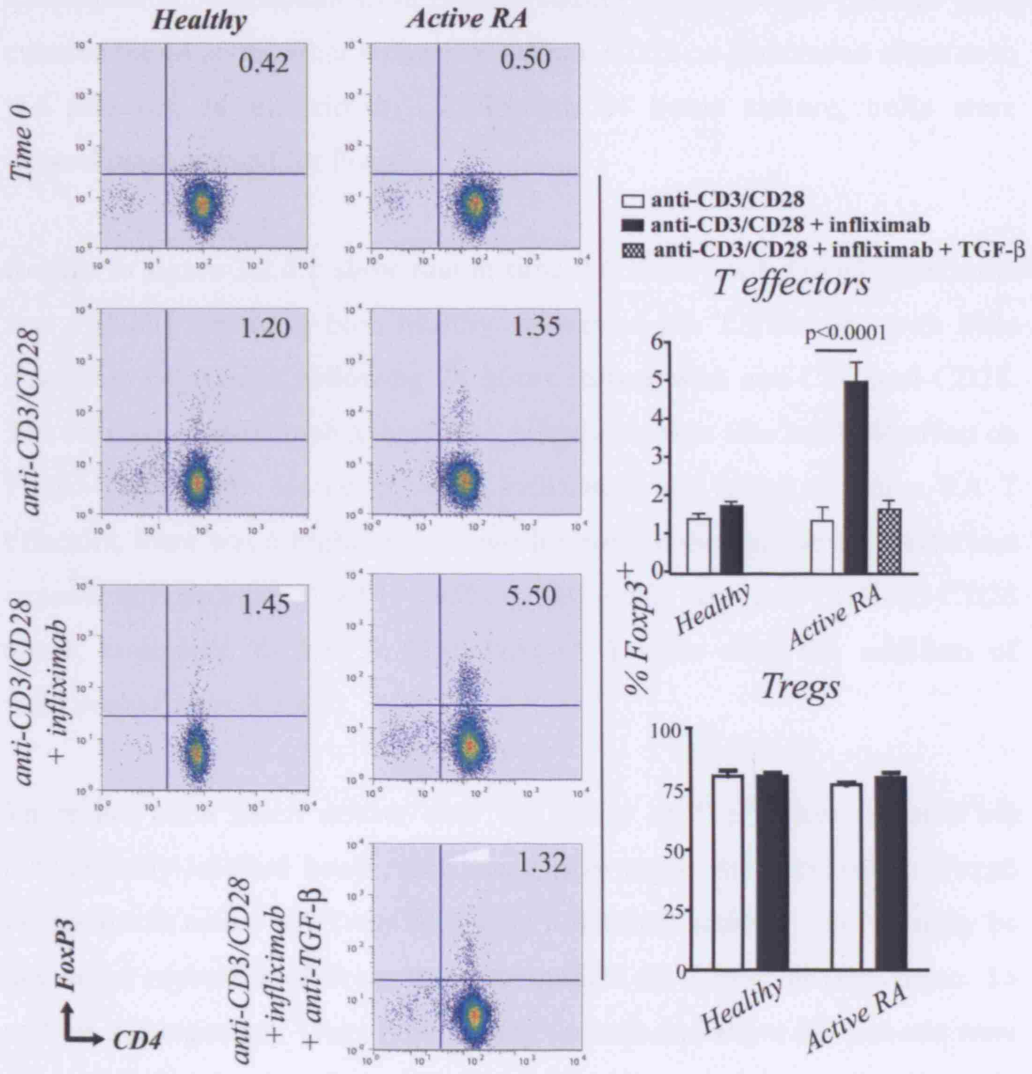


FIGURE 3.2. 4.1 IN VITRO ADDITION OF INFLIXIMAB TO ACTIVE RA T EFFECTORS INDUCES A POPULATION OF FOXP3$^+$ T CELLS

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were MACS® separated from peripheral blood of healthy controls and active RA patients. T effectors from healthy controls and active RA were stimulated with 2μg/ml of anti-CD3 and anti-CD28 ± 10μg/ml infliximab, and ± 2μg/ml anti-TGF-β. Cells were cultured for 24 hours, and then intracellularly stained for Foxp3.

Consequently, T effectors from healthy control and active RA patients were cultured for 24 hours either with anti-CD3/anti-CD28 co-stimulation alone or in the presence of infliximab. Following 24 hours culture, cells were intracellularly stained for Foxp3.

Results in figure 3.2.4.1 show that at time 0 (i.e. *ex vivo*), Foxp3 expression was virtually absent in both healthy and active RA T effectors, with little change in expression following 24 hours culture with anti-CD3/anti-CD28. The addition of infliximab to healthy T effector cultures also had little effect on Foxp3 expression. However, when infliximab was added to active RA T effectors, there was a highly significant increase in the number of T effectors expressing Foxp3 ($p < 0.0001$; 1.85% CD4+Foxp3+ with anti-CD3/anti-CD28 alone, compared to 5.80% CD4+Foxp3+ T cells after the addition of infliximab) (figure 3.2.4.1)

There has been much debate over the purity of T effectors isolated via magnetically-labelled beads, and some may argue the increase in Foxp3 expression in active RA Tregs following infliximab addition, may actually be due to the expansion of Tregs that contaminate the T effector population. To address this argument, Tregs from healthy controls and active RA patients were also isolated and cultured as above in the presence or absence of infliximab. The addition of infliximab to healthy and RA Tregs had no effect on Foxp3 expression, and levels of the Treg-specific transcription factor remained the same in both study groups (figure 3.2.4.1, bottom bar graph).

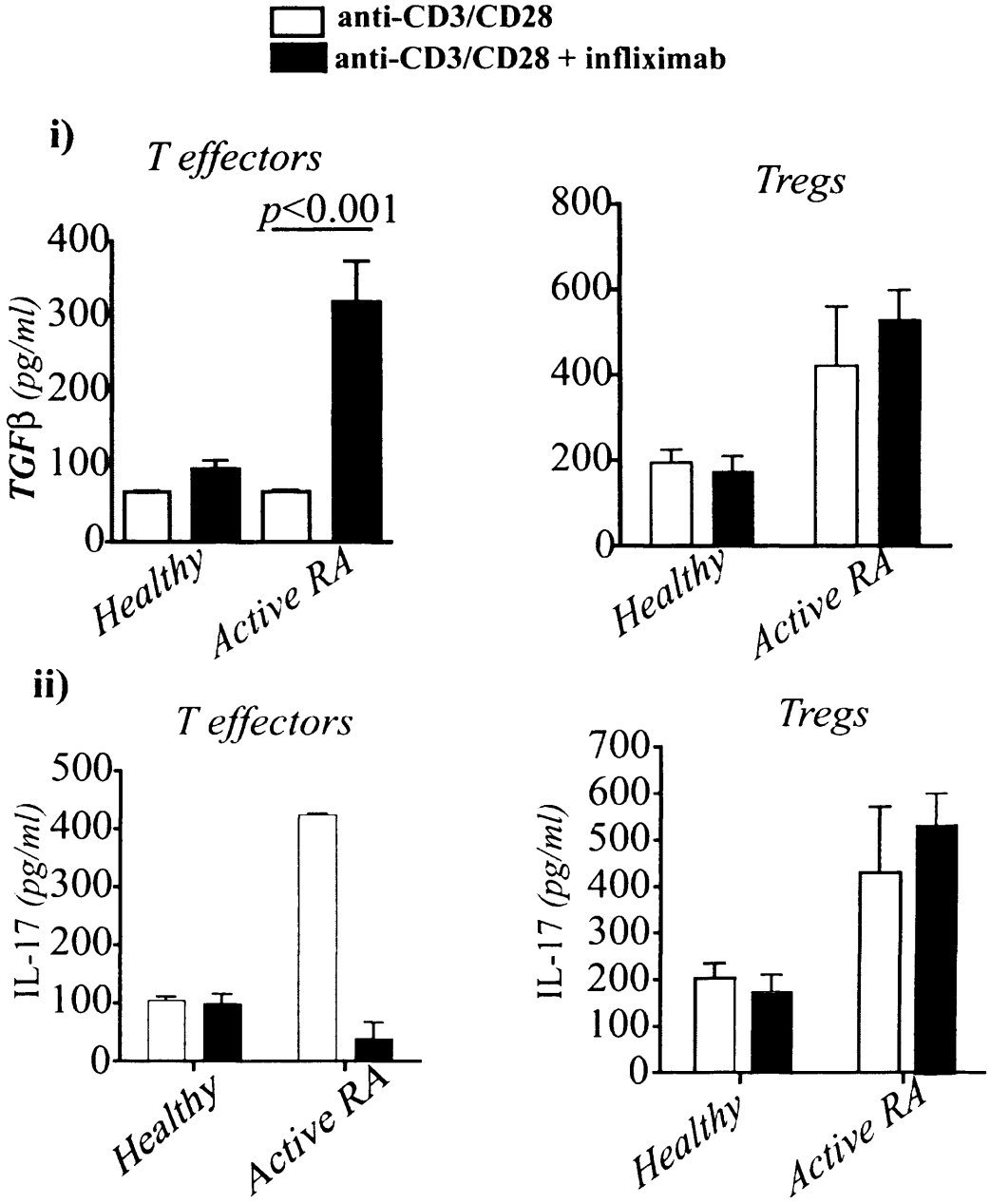
Since *ex vivo* experiments also hinted towards the involvement of TGF-β in the way $CD62L^-$ Tregs from treated patients function (but had no effect on healthy Tregs), TGF-β was also blocked in some active RA T effector cultures also containing infliximab. Analyses of Foxp3 expression in these cultures showed that the blockade of TGF-β down-regulated Foxp3 expression to levels that were similar in the presence of anti-CD3/anti-CD28 stimulation alone.

3.2.4.2 IN VITRO ADDITION OF INFLIXIMAB TO ACTIVE RA T EFFECTORS PROMOTES TGF-β PRODUCTION AND INHIBITS IL-17

The involvement of TGF-β was investigated further by looking at its production by T effectors and Tregs from active RA patients, which had been cultured with infliximab. Cells were cultured as above, and prior to staining supernatants were collected to look at TGF-β production by ELISA. Figure 3.2.4.2 i shows that healthy T effectors produce very low amounts of TGF-β regardless of the addition of infliximab. Similarly, T effectors from RA patients produced low amounts of TGF-β (50pg/ml) when cultured with polyclonal stimulation alone. However, the addition of infliximab to RA T effectors significantly increased TGF-β levels, (mean production 300 pg/ml) (figure 3.2.4.2 i, left panel). Analysis of active RA Tregs showed that these cells produced relatively high levels of TGF-β (mean production 400 pg/ml), compared to Tregs from healthy controls (mean production 150 pg/ml). The addition of infliximab to active RA Tregs did not considerably change TGF-β levels (mean 523 pg/ml), and there was no statistically significant difference in TGF-β production by active RA Tregs in the absence or presence of infliximab (figure 3.2.4.2 i, right panel).

Given the close association between TGF-β and IL-17, IL-17 was also measured in these cultures. Figure 3.2.4.2 ii shows that T effectors from healthy controls, in the absence or presence of infliximab produce very low levels of IL-17. By contrast, T effectors and Tregs from active RA patients produce significantly high levels of IL-17 (both cell types mean production 400 pg/ml). The addition of infliximab to RA T effectors potently inhibited IL-17 production (50pg/ml), but was not inhibited when infliximab was added to RA Tregs.

Taken together, these results suggest that infliximab specifically targets the T effector population from active RA patients, and adds further support that the anti-TNF-α drug does not simply rectify the Treg defect in these patients.



3.2.4.2 In vitro addition of infliximab to active RA T effectors promotes TGF-β production and inhibits IL-17

MACS® - isolated T effectors or Tregs from healthy controls and active RA patients were cultured for 24 hours as in 3.2.4.1. Supernatants were collected and were tested, by ELISA, for TGF-β (i) and IL-17 (ii). Representative of 3 experiments. Error bars represent SEM.

3.2.5 *In vitro induced Tregs via infliximab are functionally suppressive*

Although T effectors from active RA patients express Foxp3 after in vitro treatment with infliximab, it was necessary to see if these cells are functionally suppressive. T effectors from healthy controls and active RA patients, were isolated by MACS® and a fraction of T effectors were frozen down (freshly isolated T effectors). The remaining T effectors were cultured *in vitro* with anti-CD3/anti CD28 in the presence or absence of infliximab, and following 24 hour culture, these cells were separated again by MACS® in order to determine which cells are functionally suppressive i.e. the "$CD25^+$" or "$CD25^-$" population. Separated cells from the second round of purification were put back into culture at a 1:2 ratio with the freshly isolated T effectors. Cells were co-cultured for a further 48 hours, with PMA, ionomycin and Golgi plug added in the last 5 hours of culture and inhibition of TNF-α and IFN-γ was measured by flow cytometry.

Figure 3.2.5 i shows that $CD4^+CD25^+$ T cells isolated from healthy controls, from the second round of purification, cultured with anti-CD3/anti-CD28 in the presence or absence of infliximab were still unable to suppress pro-inflammatory cytokines from freshly isolated T effectors, and this was true of $CD4^+CD25^+$ T cells from active RA cultured with anti-CD3/anti-CD28 stimulation alone. However, $CD4^+CD25^+$ T cells from active RA patients that were originally cultured with infliximab significantly suppressed both TNF-α and IFN-γ production from freshly isolated autologous T effectors (figure 3.2.5 i). Interestingly, the addition of infliximab to active RA T effectors also induced a shift in the expression of CD62L in these cells from a $CD62L^+$ to a $CD62L^-$ phenotype (figure 3.2.5 ii)

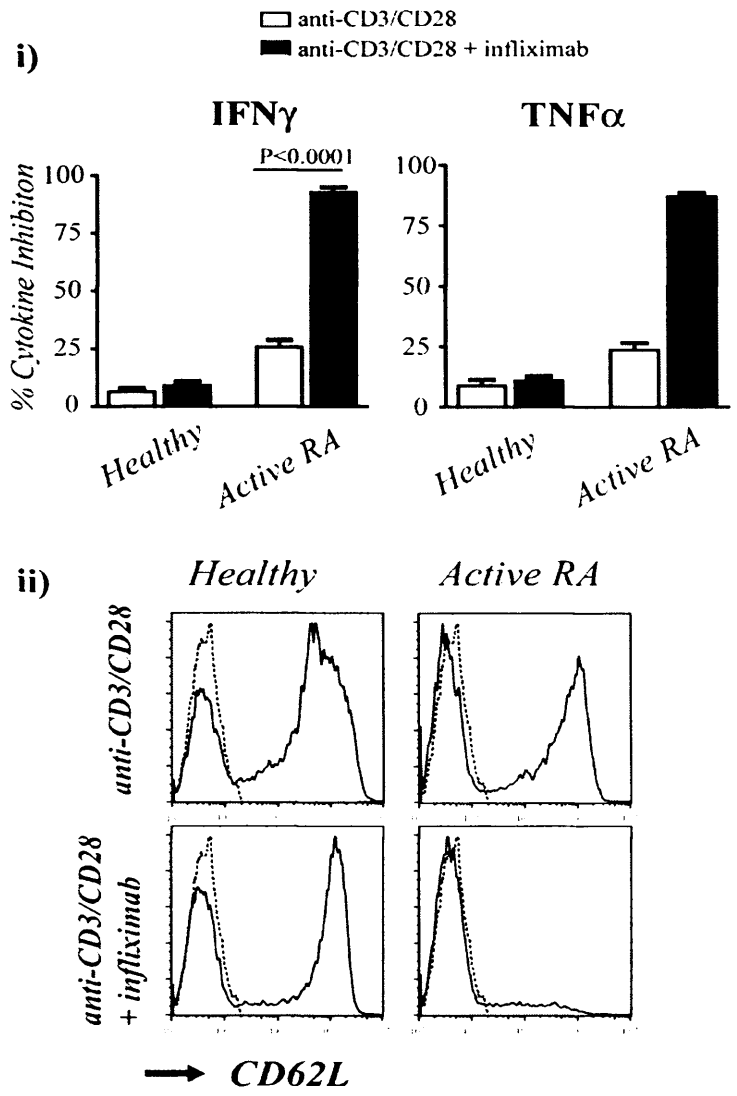


FIGURE 3.2.5 INFLIXIMAB TREGS INDUCED IN VITRO ARE FUNCTIONALLY SUPPRESSIVE

Purified T effectors from healthy controls and active RA patients were cultured as in 3.2.4 MACS® separated T effectors were stimulated with anti-CD3 and anti-CD28 in the presence or absence of 10μg/ml infliximab. Following 24 hours culture, cells were purified again by MACS® into $CD4^+CD25^-$ (and $CD4^+CD25^+$ T cells. Cells from the second round of purification were then cultured at a 1:2 ratio with freshly isolated T effectors. Cells were cultured for a further 48 hours, and surface stained for CD62L and intracellularly stained for TNF-α and IFN-γ. Results are means of three experiments and are shown as percentage cytokine inhibition. Representative histogram plots shown; dotted lines illustrate isotype controls.

3.2.6 Defective TGF-β signalling in T effectors from active RA patients

Figure 3.2.3 has shown that there is no significant difference in LAP expression on Tregs from active RA patients, when compared to healthy controls. This suggests that with regard to TGF-β, the Treg defect in RA patients does not necessarily completely lie within the Treg itself. Consequently, it was necessary to look further into the TGF-β signalling pathway. Successful TGF-β signalling relies on TGF-β binding onto its receptor, and as described in the introduction, promotes the phosphorylation of TGF-β-specific signalling molecules Smad 2 and 3.

Analysis of TGF-βRII MFI revealed that T effectors from active RA patients have significantly low levels (p= 0.0201) of receptor expression on their surface, when compared to healthy controls (figure 3.2.6 i). This suggested that the TGF-β signalling pathway might be defective in the T effectors of active RA patients. In order to investigate this hypothesis, T effectors from healthy controls and active RA patients were activated for 1.5 hours with anti-CD3 and anti-CD28 and western blot analysis was carried out on cultured T effector lysates to determine Smad2/3 phosphorylation. Figure 3.2.6 ii (top panels) show that activation of healthy T effectors resulted in normal Smad2/3 phosphorylation.

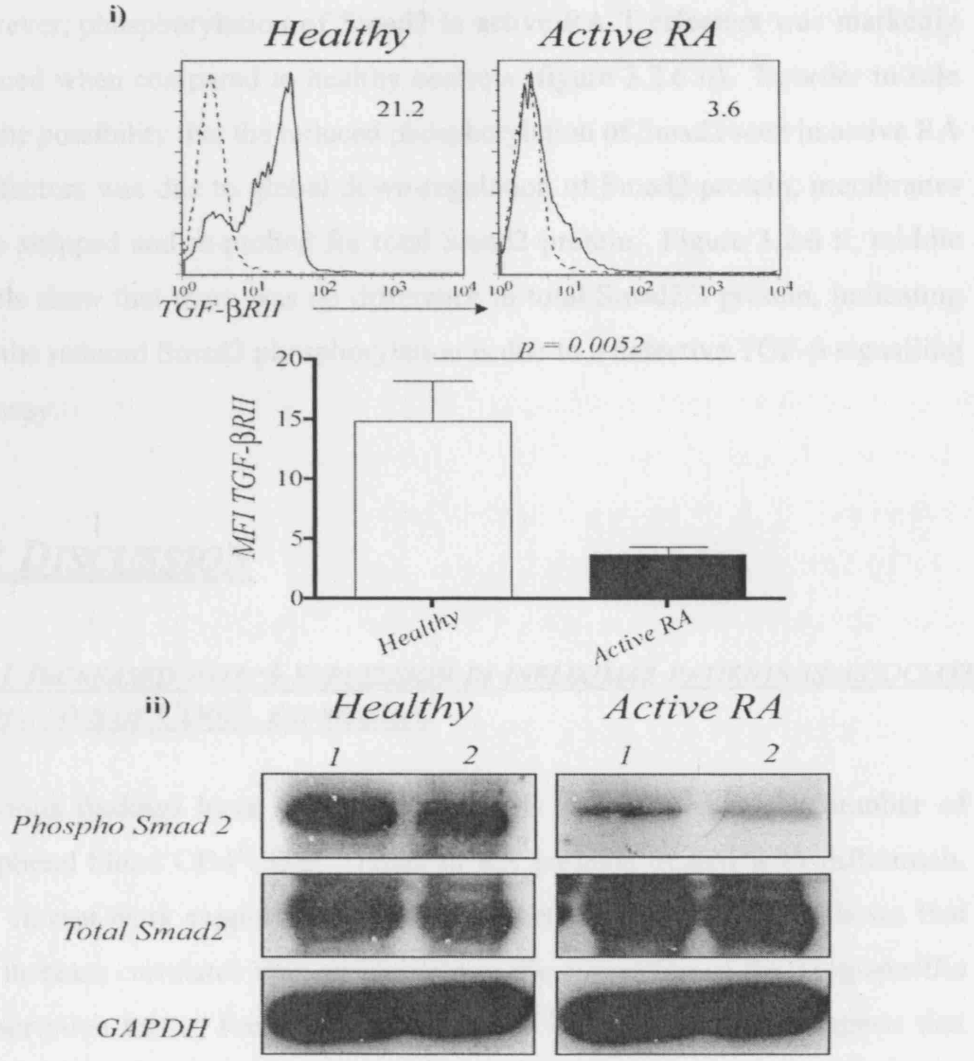


FIGURE 3.2.6 TGF-β SIGNALLING IS DEFECTIVE IN T EFFECTORS FROM RA PATIENTS.

(i) T effectors from healthy controls and active RA patients were surface stained for CD4 (APC) and TGF-βRII (PE). Histogram plots depict mean fluorescence intensity (MFI) of TGF-βRII from 3 healthy controls and 3 active RA patients. (ii) MACS-isolated T effectors from healthy controls and active RA patients were stimulated for 1.5 hours with 2μg/ml anti-CD3 and anti-CD28. Following culture, cell lysates were prepared as described in materials and methods. Western blots were carried out and membranes were probed for phosphorylated Smad 2 and total Smad 2 protein (see materials and methods). GAPDH depicts equal loading. Representative FACS and histogram plots shown; dotted lines illustrate isotype control.

However, phosphorylation of Smad2 in active RA T effectors was markedly reduced when compared to healthy controls (figure 3.2.6 ii). In order to rule out the possibility that the reduced phosphorylation of Smad2 seen in active RA T effectors was due to global down-regulation of Smad2 protein, membranes were stripped and re-probed for total Smad2 protein. Figure 3.2.6 ii, middle panels show that there was no difference in total Smad2/3 protein, indicating that the reduced Smad2 phosphorylation is due to a defective TGF-β signalling pathway.

3.2 DISCUSSION

3.2.1 INCREASED FOXP3 EXPRESSION IN INFLIXIMAB PATIENTS IS ASSOCIATED WITH A LOSS OF CD62L EXPRESSION

Previous findings have shown that there is an increase in the number of peripheral blood $CD4^{+}CD25^{hi}$ Tregs in RA patients treated with infliximab. The current work supports this finding further, where it has been shown that this increase correlates with an increase in the expression of the Treg-specific transcription factor, Foxp3. The increase in Foxp3 expression suggests that infliximab is either promoting the expansion of Tregs that are already present in the peripheral blood; or it is inducing a distinct population of Tregs that have arisen from a non-Treg population. To address these possibilities, phenotypic differences were analysed in Tregs from healthy controls, active RA patients and RA patients treated with infliximab. These analyses revealed that $Foxp3^{+}$ Tregs from healthy controls and active RA patients were predominantly $CD62L^{+}$ and $CCR7^{+}$, as well as $CD45RO^{+}$. By contrast, $Foxp3^{+}$ T cells from infliximab patients lacked CD62L, as well as CCR7 expression, but remained CD45RO positive (figure 3.2.1 i-iv).

The phenotype displayed by Tregs from infliximab patients resembles T cells of a memory T cell population ($CD62L^-$), suggesting that Tregs from these patients have been induced from an already established T cell population in the periphery. Therefore, infliximab may promote the development of an adaptive Treg population, which is phenotypically distinct from conventional Treg population that are derived from the thymus. CD62L is also a trafficking/homing molecule, and expression of CD62L on newly developed T cells in the thymus is important for their migration to the secondary lymphoid organs, particularly the lymph nodes (LNs). Tregs in human secondary lymphoid organs that have just left the thymus express CCR7. This enables them to migrate to the T cell zones, which express the CCR7 ligand, CCL19. Expression of both CD62L and CCR7 enables T cells to continuously recirculate through the lymphoid organs. However, upon T cell priming, T cells lose expression of CD62L and CCR7, which in turn, enables them to migrate to the sites of inflammation.

The reduced expression of CD62L and CCR7 suggests that infliximab may also promote the migration of Tregs to the joint by down-regulating expression of the homing molecules, and allow Tregs to actively suppress disease. Therefore, infliximab may have two complementary roles in RA therapy in relation to Tregs: first, it induces a distinct population of Tregs that lacks CD62L and CCR7, and secondly it may allow Tregs to actively migrate to the joint and inhibit inflammation. However, due to ethical constraints, as well as the lack of joint fluid following infliximab therapy, it is quite difficult to prove whether infliximab promotes the migration of Tregs into the joint.

3.2.2.1 CD62L$^-$ TREGS FROM INFLIXIMAB PATIENTS ARE MORE POTENT SUPPRESSORS AND RELY ON TGF-β AND IL-10

In order to address the question of whether infliximab induces a distinct population of Tregs it was necessary to determine whether the Foxp3$^+$CD62L$^-$ Tregs from infliximab patients were functionally suppressive. By looking at the effect on responder T cell proliferation and pro-inflammatory cytokine production, analysis of CD62L$^+$ and CD62L$^-$ Treg function from healthy controls, active RA and infliximab patients revealed three things: Firstly, in agreement with previously published findings, CD62L$^+$ Tregs from healthy controls were more potent suppressors of both responder T cell proliferation and cytokine production.

Previous findings from this lab had shown that Tregs from active RA patients are defective in terms of their inability to suppress pro-inflammatory cytokines, but are still able to suppress T effector proliferation (277). However, the current work has shown that although CD62L$^+$ Tregs were still able to suppress proliferation, the level to which they suppressed was not similar to that seen in healthy controls (figure 3.2.2.1 i). Moreover, the suppressive capacity of CD62L$^-$ Tregs from active RA patients was significantly impaired. This suggests that the discrepancy could be explained by the fact that in the original work, Tregs were sorted on just their expression of CD4 and CD25, whereas in the current work, Tregs were further sub-divided into two populations, which can further target defective Tregs from active RA patients. Moreover, due to their defective nature, the relative ineffectiveness of active RA Tregs to suppress could be due to the Tregs themselves proliferating. One way this could be addressed is to do CFSE staining. By gating on the Treg population and looking at CFSE incorporation, this will determine whether active RA Tregs are prone to *In vitro* proliferation in the presence of autologous T effectors.

In contrast to healthy controls, $CD62L^-$ Tregs from infliximab patients were more potent functional suppressors than their $CD62L^+$ counterparts. Of particular interest, $CD62L^+$ Tregs from infliximab patients resembled Tregs from active RA patients in their relative inability to suppress both T effector proliferation and pro-inflammatory cytokine production (figure 3.2.2.1. i and ii), suggesting that thymically derived Tregs from active RA patients are inherently defective, in which simply neutralising TNF-α is not enough to restore the defect. These results also suggest that factors other than TNF-α, may be responsible for the RA Treg defect, including factors such as IL-17 or defects in signalling pathways, such as TGF-β.

Taken together, these results infer that rather than reverse the defect of conventional RA Tregs, infliximab induces a population of Tregs from a non-Treg population that are sufficient in number to over-ride the defective $CD62L^+$ Treg population. Indeed, a very recent report has shown that the addition of the immunosuppressive drug FK778 is capable of inducing Tregs from $CD4^+CD25^-$ T cells. FK778 is currently being developed for use in organ transplantation, and is a synthetic analogue of the active metabolite of leflunomide, A77 1726, which is used in the treatment of RA. Leflunomide, as well as its synthetic analogues, is a pyrimidine synthesis inhibitor, and similar to infliximab, leflunomide has been shown to inhibit TNF-α signalling (278).

Like its parent drug, FK778 inhibits T cell activation, and in addition induces Tregs from the T effector population, the authors have demonstrated that the FK778-induced Tregs displayed a memory-like phenotype, where the Tregs expressed Foxp3, but lacked CD62L expression (279). The findings from this paper and the results already discussed, suggest that the inhibition of TNF-α,

commonly seen in a disease setting, promotes the induction of Tregs. It should be noted that the induction of $Foxp3^+$ Tregs is not a generalised observation following all anti-TNF-α therapies. Analysis of etanercept patients showed no increase in peripheral blood Tregs (figure 3.1.1.1).

This implies that the potential for anti-TNF-α drugs to induce suppressor T cells depends on its mode of action. Etanercept functions by binding to soluble TNF-α, whereas infliximab physically binds onto the cell, preventing TNF-α from binding. The implication of infliximab binding to the target cell (T cell, monocyte) may mean that the drug is affecting down-stream molecular pathways that can change the suppressive capability of the cell.

Since it had been demonstrated that $CD62L^-$ Tregs from infliximab patients were functionally better suppressors than their $CD62L^+$ counterparts, and therefore supporting the hypothesis that the $CD62L^-$ Tregs were induced by infliximab, it was necessary to determine how these Tregs function. Both IL-10 and TGF-β have been implicated in the function of induced/adaptive Tregs, and consequently, these cytokines were neutralised in co-cultures of T effectors from healthy controls and infliximab patients, containing $CD62L^+$ and $CD62L^-$ Tregs. The cytokines were either blocked on their own or together, in the presence of polyclonal stimulation.

Neutralisation of TGF-β ands/or IL-10 in cultures containing $CD62L^+$ or $CD62L^-$ Tregs from healthy controls did not affect the functional capacity of these Tregs to suppress (figure 3.2.2.2, left panels). By contrast, neutralisation of TGF-β in cultures containing $CD62L^-$ Tregs from infliximab patients, significantly abrogated their ability to suppress pro-inflammatory cytokine from the T effector population. Neutralisation of IL-10 also abrogated $CD62L^-$ mediated suppression, but not to the levels seen after TGF-β blockade. Interestingly, blockade of both cytokines in the same culture inhibited $CD62L^-$ Treg suppression at much greater levels than TGF-β blockade alone (figure 3.2.2.2 right panels, white bars). These results suggested that TGF-β and IL-10

play differing roles in $CD62L^-$ Treg-mediated suppression. The effectiveness of TGF-β blockade on inhibiting $CD62L^-$ Treg suppression suggests that this cytokine is directly involved in the way these Tregs function.

However, the comparative ineffectiveness of IL-10 blockade alone to inhibit $CD62L^-$ Treg function suggests that this cytokine may be required for the induction of TGF-β, rather than the function of this subset of Tregs. This has been shown in murine models of colitis. Both TGF-β and IL-10 have been demonstrated to be important in protecting mice from the disease. However, while TGF-β demonstrated a direct role in Treg-mediated suppression, IL-10 was not directly involved. Instead, it was shown that IL-10 was important in driving the production of TGF-β, and therefore important in the maintenance of TGF-β- producing Tregs (280). This model would fit in with the conditions induced by infliximab: the inhibition of TNF-α would promote an increase in IL-10 production, which in turn up-regulates TGF-β production which is necessary for the development and function of induced Tregs.

However, it could be argued that if this model is true, then it should also be observed in etanercept patients. One possibility that could explain this is that etanercept patients may have an increased number of $Foxp3^-$ Tr1 (IL-10-producing) cells, which could account for the observation that these patients do not have increased levels of $Foxp3^+$ T cells (figure 3.1.4 i), and it would be interesting to see if these patients have increased levels of IL-10.

Neutralisation of TGF-β and/or IL-10 in cultures containing $CD62L^+$ Tregs from infliximab patients produced differing results to those seen in $CD62L^-$ cultures. The blockade of TGF-β appeared to have reversed the relative inability of $CD62L^+$ Tregs to suppress both TNF-α and IFN-γ (figure 3.2.2.2, right panels, black bars). This could be explained by combined inhibition of TNF-α and TGF-β, which in turn, can inhibit the production of IL-17. Indeed,

analysis of IL-17 production from T effectors, $CD62L^+$ and $CD62L^-$ Tregs from infliximab patients revealed that $CD62L^+$ T regs produce IL-17 (figure 3.2.2.2, lower panel, black bars), which is inhibited upon neutralisation of TGF-β and/or IL-10. In this context, inhibition of IL-10 would result in reduced levels of TGF-β, which in turn, together with reduced levels of TNF-α leads to reduced levels of IL-17, allowing $CD62L^+$ Tregs to suppress.

These results therefore suggest that TGF-β and IL-10 have differing effects on $CD62L^+$ "diseased" Tregs and $CD62L^-$ "healthy" Tregs. These results also suggest that blockade of TNF-α alone is not sufficient to restore the Treg defect in active RA patients, and requires inhibition of other pro-inflammatory cytokines implicated in RA pathology, namely IL-17.

3.2.3 $CD62L^-$ TREGS FROM INFLIXIMAB PATIENTS EXPRESS HIGH LEVELS OF TGF-β ON THEIR SURFACE & PROMOTE IL-10 PRODUCTION

The neutralisation experiments suggest that $CD62L^-$ Tregs from infliximab patients function in a cytokine-dependent manner. However, previous findings from this lab have suggested that Tregs from these patients function in a cell contact-dependent fashion. Although the findings in the previous and current work appear conflicting, they can be explained by the presence of TGF-β. TGF-β can mediate suppression via cell contact. Tregs isolated from mice have been shown to produce both TGF-β and IL-10; however, the cells only suppressed in a cell contact-dependent manner. These Tregs also expressed high levels of TGF-β on their surface, which, by binding to TGF-β receptor (measured via TGF-βRII) on T effectors, were able to induce cell contact-dependent suppression (131). Subsequent studies by the same group have shown that human Tregs function is also dependent on cell-surface bound TGF-β, which can be detected in the form of LAP (281).

Figure 3.2.3 i shows that while Tregs from healthy controls and active RA patients express some LAP on their surface, there is a significant up-regulation of surface-bound TGF-β on Tregs isolated from infliximab-treated patients. The increased LAP expression, coupled with the observation that $Foxp3^+$ T

cells from infliximab patients have low CTLA-4(figure 3.1.4.1) suggest that although $CD62L^-$ Tregs function via cell contact, they do so in a different way to conventional Tregs. $CD62L^-$ Tregs from infliximab patients function via increased cell-bound TGF-β, which may compensate for the reduced levels of CTLA-4.

The observation that cultures containing $CD62L^-$ Tregs from infliximab patients promote the production of IL-10 (figure 3.2.3 ii), adds further support to the neutralisation experiments, that demonstrate that both IL-10 and TGF-β are implicated in the induction and function of $CD62L^-$ Tregs in infliximab patients. However, although it is tempting to call these $CD62L^-$ Tregs Tr1 cells, the nature of the culture experiments looking at IL-10 does not define which cells are actually making the cytokine. It could be that the $CD62L^-$ Tregs themselves are making IL-10, or are promoting T effectors to make IL-10, which in turn induces TGF-β in the Tregs.

3.2.4.1 In vitro addition of infliximab to active RA T effectors induce a population of Foxp3$^+$ T cells

It was necessary to determine further, whether a), infliximab directly induces a population of Tregs from the T effector population, and b), how these Tregs function. In order to address these questions, *in vitro* experiments were carried out in which T effectors from both healthy controls and active RA patients were cultured with soluble anti-CD3/anti-CD28 in presence or absence of infliximab for 24 hours (figure 3.2.4.1).

The expression of Foxp3 was measured at time 0 in both healthy and RA T effector populations, and showed that both groups expressed very little Foxp3. Following 24 hours culture, no difference was seen in Foxp3 expression in T effectors from both healthy controls and active RA patients in the presence of anti-CD3/anti-CD28. However, it has been demonstrated that Foxp3 is induced in T effector populations following 24 hours culture with anti-CD3/anti-CD28. However, the authors used to plate-bound anti-CD3 at 5μg/ml together with 1μg/ml soluble anti-CD28, whereas in the current work only 2μg/ml of both

soluble anti-CD3 and anti-CD28 were used. Plate-bound anti-CD3 provides a much greater stimulus for T cell activation that is enhanced further in the presence of anti-CD28. This may explain the differing results obtained, and therefore suggests that potency of stimulation affects Foxp3 expression.

Analysis of Foxp3 expression in the presence of anti-CD3/anti-CD28 plus infliximab revealed that there was no induction of the transcription factor in T effectors from healthy controls. However, the presence of infliximab in cultures containing RA T effectors resulted in a significant induction of Foxp3. Interestingly, the addition of infliximab to RA Tregs had no effect on Foxp3 expression, adding further support that infliximab is not simply rectifying the Treg defect seen in RA patients.

The differential effect of infliximab on T effectors from healthy controls and active RA patients suggest that TNF-α plays an important role in determining *de novo* Foxp3 expression. RA patients have reduced CD28 expression on their $CD4^+$ T cells due to the presence of TNF-α (282). Two recent studies have demonstrated that CD28-mediated co-stimulation is required for the development of Tregs.

The first of these papers shows that expression of Foxp3 in developing thymocytes is dependent on CD28 co-stimulation (283). The second of these papers shows that CD28 co-stimulation is required for TGF-β-mediated conversion of $CD4^+CD25^-$ T cells to Foxp3-expressing cells (284). This may explain the effect infliximab has on active RA T effectors: by actively blocking/inhibiting TNF-α, CD28 levels can return to normal and in turn T effectors can respond to normally regulated TCR stimulation, allowing TGF-β mediated induction of Tregs.

Because inhibition of TGF-β from *ex vivo* cultures of $CD62L^-$ Tregs from infliximab patients abrogated their suppressive activity, TGF-β was also blocked in *in vitro* cultures containing RA T effectors in the presence of

infliximab. Blockade of TGF-β resulted in a down-regulation of Foxp3 expression, to levels similar to that of T effectors at time 0.

3.2.4.2 IN VITRO ADDITION OF INFLIXIMAB TO ACTIVE RA T EFFECTORS PROMOTES TGF-β PRODUCTION AND INHIBITS IL-17

The presence of infliximab in RA T effector cultures, but not healthy controls, resulted in the production of TGF-β, suggesting that blocking TNF-α results in the induction of TGF-β, which subsequently leads to Foxp3 expression. Given the close association between TGF-β and IL-17, it seemed reasonable to investigate the production of IL-17 under the above culture conditions. ELISAs for IL-17 demonstrated that the cytokine is produced low amounts from T effectors of healthy controls in the presence or absence of infliximab (figure 3.2.4.2 ii). Interestingly, T effectors from active RA patients produced high levels of IL-17, which were diminished in the presence of infliximab. The inhibition of TNF-α via infliximab is likely to have a cascade effect on other pro-inflammatory cytokines, including IL-17, IL-1 and IL-6. This would create an environment for TGF-β, which can now act as an anti-inflammatory cytokine, enabling it to promote the induction, maintenance and function of $Foxp3^+$ Tregs from active RA T effectors.

Analysis of Tregs from active RA patients revealed that they produce high levels of TGF-β, which did not change significantly with the *in vitro* addition of infliximab. Similarly, active RA Tregs produced high levels IL-17. Moreover, similar to Foxp3 expression, the addition of infliximab did not inhibit production of Il-17 (figure 3.2.4.2 ii). This is interesting because the addition of infliximab to active RA T effectors was able to inhibit IL-17 production. Taken together, the differing results observed between RA Tregs and T effectors, in terms of Foxp3 expression and IL-17 inhibition, suggest that active RA Tregs are likely to be in a committed defective state, which is

confirmed by the inability of $CD62L^+$ Tregs from infliximab patients to suppress cytokine production, and simply blocking TNF-α is insufficient to overcome their defective state.

In light of the above results, it would be intriguing to investigate whether blocking TGF-β together with TNF-α is sufficient to overcome the defect, as has been observed for $CD62L^+$ Tregs in infliximab patients (figure 3.2.2.2 and ii). Recently, it has been shown that $Foxp3^+$ Tregs can be self-induced or are able to induce T effectors to become Th17 cells in the absence of exogenous TGF-β, but in the presence of Treg-derived TGF-β and exogenous IL-6 (285). Active RA Tregs express LAP on their cell surface at levels similar to that seen in healthy controls (figure 3.2.3 i). This may explain the cytokine profile observed in active RA Tregs and T effectors, together with the cytokine profile of both subsets of T cells are exposed to as a result of RA (e.g. increased levels of IL-6): active RA Tregs are defective since they may actually be Th17 cells, and rather than suppress T effectors, they induce T effectors to become IL-17 producing T cells.

3.2.5 In vitro induced Tregs via infliximab are functionally suppressive

Although in vitro addition of infliximab resulted in the induction of Foxp3, this did not necessarily imply that the induced $Foxp3^+$ T cells are functionally suppressive. Pre-incubation of T effectors from healthy controls or RA patients with infliximab (plus polyclonal activation) showed that infliximab-primed T cells from active RA patients were also functionally suppressive. More specifically, further separation of the incubated cells into $CD4^+CD25^-$ and $CD4^+CD25^{hi}$, revealed that only the latter population were capable of suppressing cytokine production from freshly isolated T effectors, and that these infliximab-induced $CD4^+CD25^+$ T cells acquire a $CD62L^-$ phenotype. |These results confirm that infliximab is able to induce a distinct population of $CD4^+CD25^{hi}$ Tregs that differ phenotypically from natural Tregs in terms of

CD62L expression, but express Foxp3. The low expression of CD62L and CCR7 on these cells suggests that they are distinct from the adaptive Tr1 or Th3 cells previously described but others. Instead, infliximab appears to induce functionally suppressive T cells from peripheral T effector populations in active RA patients.

3.2.6 TGF-β SIGNALLING IS DEFECTIVE IN T EFFECTORS FROM ACTIVE RA PATIENTS

The results discussed above regarding IL-17 production from active RA Tregs, suggests one possibility why Tregs from these patients are defective. Another possibility might be that the T effectors are not able to receive inhibitory signals properly from the Treg. As already shown in figure 3.1.1, $Foxp3^+$ Tregs from active RA *and* infliximab patients have significantly lower levels of CTLA-4, compared to healthy controls, and this could also explain the RA Treg defect. However, increasing evidence suggests that conventional Tregs function via TGF-β in the periphery, in a cell contact-dependent fashion (131). Therefore, the RA Treg defect could be explained either by reduced levels of TGF-β on their surface, or defective TGF-β signalling in RA T effectors.

To explore this hypothesis, Tregs and T effectors from healthy controls and active RA patients were isolated via MACS®. Figure 3.2.3 i shows that both healthy controls and active RA Tregs express similar levels of LAP, suggesting that the RA Treg defect, in terms of TGF-β, is not due to decreased levels of cell surface-bound TGF-β. Analysis of RA T effectors revealed two striking observations. Firstly, T effectors from RA patients have significantly lower MFIs of TGF-βRII on their surface (figure 3.2.6 i). Secondly, phosphorylated Smad2/3, but not total Smad2/3 protein, is down regulated in active RA T effectors (figure 3.2.6 ii). These results suggest that T effectors from active RA patients have a defective TGF-β signalling pathway. A recent paper has demonstrated that T cells from mice that are transgenic for a truncated TGF-βRII (dnTGF-βRII) are refractory to Treg-mediated suppression and consequently, these mice experience increased colitis-related pathology (286). The authors from this paper suggest that T cells from these mice escape control

from Tregs because they cannot respond to TGF-β. Similarly, the results discussed here might also explain the RA Treg "defect": T effectors from active RA patients are refractory to Treg suppression due to a defective TGF-β signalling pathway.

In spite of the above results, previous published findings from this lab have shown that T effectors from active RA patients are susceptible to suppression by Tregs from the same patients following infliximab therapy (255), suggesting that defect does not lie within the T effector population. However, one explanation for the discrepancy between the current and previously published findings could be that the Tregs from infliximab patients are capable of restoring the T effector defect. For example, the high levels of TGF-β on the surface of infliximab-treated RA Tregs, together with the ability of $CD62L^-$ Tregs to promote IL-10 production, could promote the up-regulation of TGF-β receptor and normal phosphorylation of Smad signalling molecules. Consequently, it will be interesting to investigate whether normal Smad phosphorylation and TGF-βRII expression is induced in active RA T effectors following co-culture with Tregs of the same patients after infliximab therapy.

CHAPTER 4

FINAL DISCUSSION AND CONCLUSIONS

4.1 FINAL DISCUSSION

The data presented in this thesis has investigated Treg phenotype and function, comparing patients with active RA with those treated with the anti-TNF-α drug, infliximab. Analysis of Tregs from these patients revealed striking differences in phenotype: in contrast to healthy controls and infliximab-treated patients, active RA Tregs express low levels of the chemokine receptor CCR5, but express significantly higher levels of CD40L. Furthermore, analyses of cytokines produced by Tregs from active RA patients have demonstrated that these cells express normal levels of cell-bound TGF-β (in the form of LAP), when compared to healthy controls, but produce increased levels of soluble TGF-β, as well high levels of IL-17.

Another major finding is the induction of a distinct population of Tregs in RA patients following infliximab therapy. These Tregs also differ phenotypically, in that they lack CD62L expression – a molecule that is closely associated with thymically derived Tregs. This finding is interesting, since it suggests that infliximab does not simply restore the defective state of active RA Tregs. In fact, $CD62L^+$ Tregs from infliximab patients remain functionally defective, as well as producing similar levels of IL-17 to active RA Tregs.

In addition to the obvious therapeutic advantages of an induced population of functionally suppressive Tregs via infliximab, their induction could provide an *in vitro* diagnostic tool to predict whether an active RA patient will be a successful responder to anti-TNF-α therapy (since not all RA patients successfully respond). This idea stems from the fact that increased numbers of Tregs were only observed in RA patients that responded to infliximab, but not non-responders (255), and that *in vitro* addition of infliximab led to the induction of $Foxp3^+$ Tregs in active RA patients, but not healthy controls.

The change in phenotype in active RA Tregs may explain their defective suppressor function, which this lab has previously shown (255). RA is typically characterised as a Th1-mediated autoimmune disease. However, Th17 cells have recently been characterised as a distinct IL-17 –secreting T helper cell subset, and blockade of IL-17 has proven effective in animal models of RA, such as CIA (287-289). These *in vivo* experiments, therefore suggest that RA may actually skew towards being a Th17-mediated disease, rather than Th1.

The development of Th17 cells has been closely associated, at least in murine studies, with TGF-β in the presence of a pro-inflammatory environment. Moreover, studies have shown that stimulation of naïve human T cells with TGF-β leads to the up-regulation of CD40L, as well as TNFR2 (290). However, the stimulatory effect of TGF-β was found to be concentration dependent, where lower concentrations (10-1000 pg/ml) were stimulatory, whereas higher concentrations were inhibitory (290). Analysis of TGF-β production by active RA Tregs, (as wells as active RA T effectors) demonstrated that the mean production was around 420 pg/ml (figure 3.2.4.2 i). This concentration of TGF-β lies within the range of the published results describing the co-stimulatory effect of TGF-β. Therefore, this may provide a mechanism to explain the high levels of CD40L expressed on active RA Tregs.

Recently it has been demonstrated that human Th17 cells can be characterised based on their lack of CCR5 expression (291). The authors from this paper show that the T cells that do not express CCR5 produce large amounts of IL-17, thereby distinguishing Th17 cells from Th1 cells. The low levels of CCR5 on active RA Tregs, coupled with their ability to produce significant levels of IL-17, suggest that active RA Tregs could be IL-17-producing T cells. Given that the developmental pathway of Tregs and Th17 is extremely close (see figure 1.2.2.3), this may indicate that Tregs from active RA patients are intrinsically defective.

If active RA Tregs are intrinsically defective, then this should also apply to active RA T effectors, since both subsets of cells are exposed to the same pro-inflammatory conditions. However, the addition of infliximab to RA T effectors, but not to RA Tregs down-regulates IL-17 production and induces Foxp3 expression. Moreover, the addition of infliximab increases soluble TGF-β production. Analyses of CCR5 and CD40L on T effectors from active RA patients demonstrated that levels were comparable to T effectors from healthy controls and infliximab-treated patients: T effectors from all three study groups expressed relatively high levels of CCR5, but low levels of CD40L.

This could explain the difference observed between Tregs and T effectors from active RA patients when both subsets of cells are cultured *in vitro* with infliximab. During the process of naive CD4+ T cell differentiation, they acquire reciprocal sets of chemokine receptors. For example, differentiating Th1 cells preferentially express CCR5, whereas Th2 cells express CCR4 (292). The low expression of CCR5 observed on active RA Tregs, together with the pro-inflammatory environment to which they are exposed, as well as augmented production of soluble TGF-β, suggest that these cells may develop along the Th17 pathway. In other words, active RA Tregs are "programmed" to be defective, whereas T effectors are not.

Studies looking at human renal epithelial cells have shown that T cell derived IL-17 and CD40L work in synergy to enhance IL-6 production. The authors also show that IL-17 up regulates CD40 expression on renal epithelial cells (293). IL-6 is a key determining factor in the Treg/Th17 pathway, and the high IL-17 production, coupled with high CD40L expression on active RA Tregs could provide a positive feedback loop that maintains their Th17-like phenotype, which is still seen in $CD62L^{+}$ Tregs from infliximab-treated RA patients.

Recent studies have shown that Tregs can present a TGF-β signal to T effectors via cell-cell contact, which is independent to soluble TGF-β (294). In addition, these findings support previous studies showing that the surface TGF-β on Tregs is in a form that is capable of being activated when it is presented to its receptor at the point of cell-cell contact (131). Cell surface CTLA-4 cross-linking has been shown to result in accumulation of TGF-β at the site where suppressor cell-target contact and CTLA-4-B7 ligation occurs (294). Intracellular flow cytometric analysis revealed that CD4$^+$Foxp3+ Tregs from active RA patients express significantly low levels of CTLA-4, and hence reduced CTLA-4 cross-linking. This dependence of CTLA-4 in bringing TGF-β to the point of cell contact may explain the defective Smad 2 phosphorylation observed in active RA T effectors: reduced levels of CTLA-4 results in insufficient levels of TGF-β at the point of Treg to T effector contact.

Although active RA Tregs express normal levels of TGF-β on their surface (compared to healthy controls), the current data do not show whether there is reduced TGF-β at the point of cell contact between Treg and T effector. It would, therefore, be interesting to investigate, via confocal microscopy, whether there is reduced TGF-β at the point of cell contact, and see if this corresponds to the reduced CTLA-4 in active RA Tregs.

The effect of CTLA-4 signalling on TGF-β at the point of cell contact may explain the finding that Tregs from CTLA-4 deficient mice express more surface TGF-β and IL-10 and exhibit a more TGF-β/IL-10-dependent suppression than Tregs from wild-type mice (46). This would follow from the possibility that the population of Treg cells that develops in CTLA-4-deficient mice express higher amounts of surface TGF-β to compensate for their lack of ability to promote surface TGF-β mediated by CTLA-4. Similar to CTLA-4 deficient mice, compensatory mechanisms could take place in RA patients following infliximab therapy: low levels of CTLA-4 could trigger alternate pathways for the induction of Tregs that do not rely on CTLA-4, but instead function via TGF-β and IL-10 (see figure 4.1).

The data presented in this thesis suggest that blockade of TNF-α is responsible for a reversal in the differing Treg phenotype observed in active RA patients, as well as inducing a population of functionally suppressive Tregs. This suggests that infliximab may not simply neutralise TNF-α, but also indirectly neutralises a range of pro-inflammatory cytokines and molecules, including IL-17 and CD40L.

4.2 CONCLUSIONS

The aim of this PhD was to investigate further previously published findings from this lab that had shown Tregs from patients with active RA are functionally defective. The current work presented in this thesis provides evidence that Tregs from patients with active RA differ phenotypically to Tregs from healthy controls and RA patients receiving infliximab therapy. Although the data do not directly demonstrate a link between phenotype and the defective suppressive function of Tregs from active RA patients, they do suggest a possible mechanistic explanation as to why they are unable to suppress T effector functions.

The data presented here also confirm the previous findings that Treg function is restored in RA patients following infliximab therapy. Moreover, the data demonstrate that rather than simply rectify the Treg defect seen in RA infliximab induces a distinct population of functionally suppressive Tregs that differ from conventional thymically derived Tregs, both phenotypically and mechanistically.

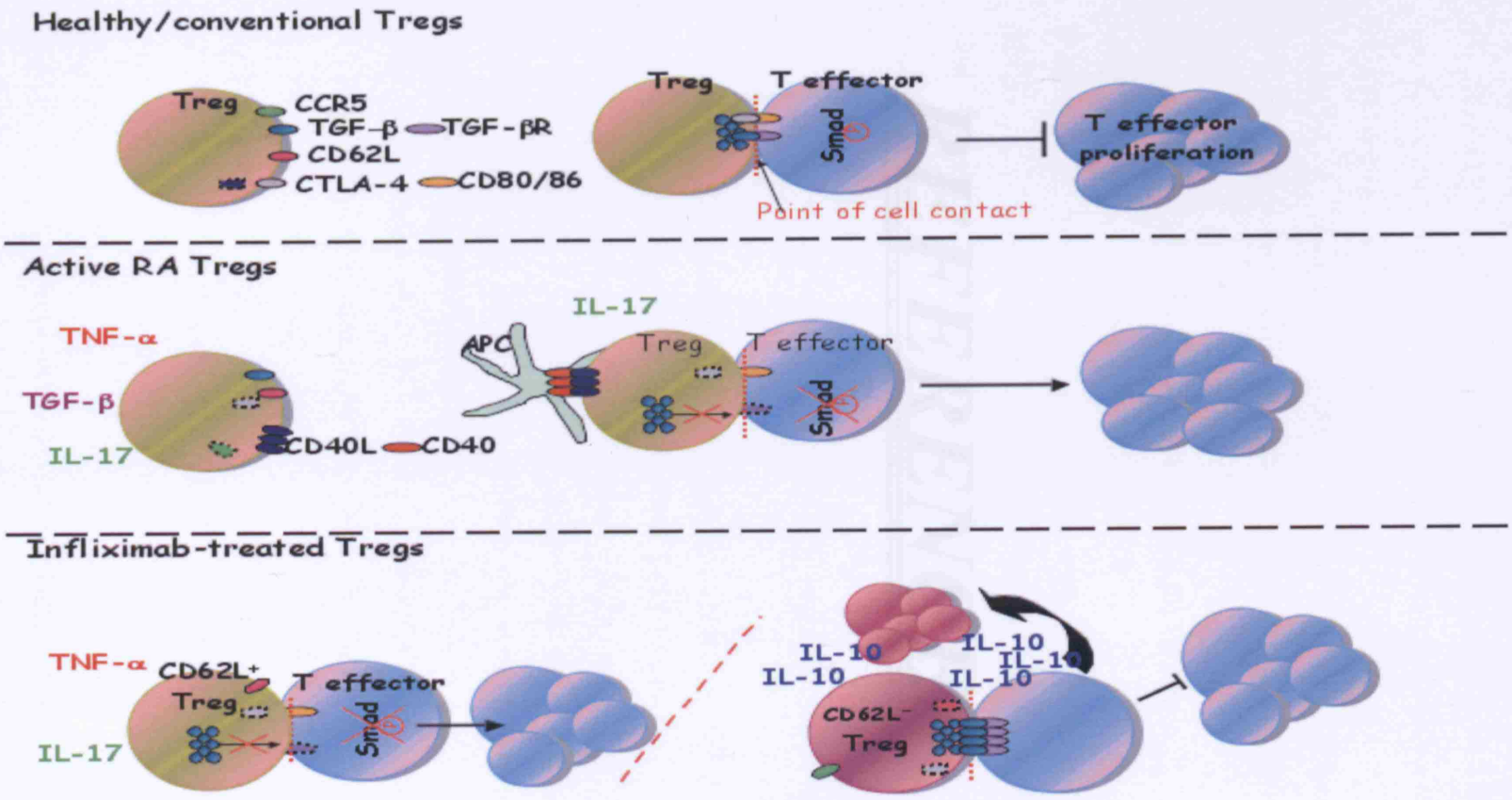


FIGURE 4.1 SUMMARY DIAGRAM OF PROPOSED MECHANISM OF ACTION OF DEFECTIVE ACTIVE RA TREGS AND FUNCTIONAL INDUCED CD62L⁻ TREGS FROM INFLIXIMAB-TREATED RA PATIENTS

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APPENDIX I: PATIENT CONSENT FORM AND SAMPLE PATIENT DATA

Royal Free and University College Medical School
UNIVERSITY COLLEGE LONDON

WINDEYER INSTITUTE OF MEDICAL SCIENCES

DEPARTMENT OF IMMUNOLOGY & MOLECULAR PATHOLOGY

Windeyer Building, 46 Cleveland Street, London W1T 4JF

Telephone 020 7679 xxxx
Fax 020 7679 xxxx
Email: xxxxxx
http://www.ucl.ac.uk/windeyer-institute

Project Title: The function(s) of regulatory T cells in rheumatoid arthritis

Principal Investigator: Dr M Ehrenstein

Description of Project: Investigation into function(s) of regulatory T cells in the above mentioned patients

Ethical Approval No: 02/0240

Confidentiality is strictly maintained at all times. Your name will not be known to anyone other than the staff immediately involved in collecting the sample. All data will be anonymous and untraceable to the volunteer except by those immediately involved in collecting the sample.

Please read and sign the following declaration:
I confirm that I have read and understood the information relating to this study. I have had the opportunity to ask questions and I understand that I can withdraw from this study at any time without giving a reason.

Signature of volunteer:..... Print:.....

Date:.....

Signature of person taking blood:..... Print: .....

Date:.....

Patient hospital Number	Type of patient	CRP	DAS
M/347497	Active RA	16.9	6.01
97065506	Active RA	32.9	6.39
M/356901	Active RA	10.1	7.12
94024383	Active RA	21.7	6.89
91105472	Active RA	42.7	7.9
92005352	Active RA	13.8	5.2
M/1376040	Infliximab	4.6	2.45
96022807	Infliximab	1.3	4.22
M/1442987	Infliximab	4.3	2.28
M/426832	Infliximab	1.3	6.27
92045278	Infliximab	4.8	6.02
M/460063	Etanercept	4.8	1.89
95049864	Etanercept	2.2	5.5
M/437745	Etanercept	4.7	6.78

APPENDIX II: REAGENTS AND BUFFERS

All general reagents from Sigma unless otherwise stated.

CELL ISOLATION AND CULTURE

PHOSPHATE BUFFERED SALINE (PBS)

Dulbecco's PBS without calcium or magnesium. X 10 sterile stock solution (Biowhittaker). Diluted in sterile endotoxin free distilled water (Biowhittaker).

TRYPAN BLUE (0.4% STOCK)

0.1% Trypan Blue (Sigma) diluted in PBS

Cells diluted 1/2 in 0.1% Trypan Blue for viability counts.

COMPLETE RPMI 1640 MEDIUM

RPMI 1640 medium with Glutamax (Life Technologies Ltd)

10% foetal calf serum (FCS) (Life Technologies Ltd)

100U/ml penicillin and streptomycin (Penstrep)

MACS BUFFER

For 1 litre of sterile MACS buffer, add 4 mls of EDTA and 5 mls of foetal calf serum to 1 X PBS

FLOW CYTOMETRY

WASHING AND STAINING BUFFER

PBS with 0.1% Sodium azide
1% Foetal calf serum (Life Technologies Ltd)
Stored at 4°C for 2 days.

FIXING BUFFER

2% paraformaldehyde diluted in PBS, 0.1% sodium azide
Stored at 4°C, protected from light for 1 week.

ELISA

WASHING BUFFER(PBS/TWEEN)
0.05% Tween-20 (polyoxyethylene sorbitan monolaurate) in PBS

BLOCKING BUFFER
2% BSA in PBS/tween,

ASSAY BUFFER
1 X PBS,
0.05% Tween-20
2% bovine serum albumin (BSA).

WESTERN BLOT

CELL LYSIS BUFFER

For 5ml stock solution:

500µls 0.5M Tris HCl pH8
500µls 1M NaCl
500µls 0.1M $MgCl_2$
50µls 1% Triton-X-100

+ 3.9mls tissue culture water

Protease and phosphatase inhibitors added just prior to use:

1mM $NaVO_4$: 50µls from 1000X stock (see below)

10mM NaF: 50µls from 100X stock (see below)

Protease inhibitor cocktail: 50µl from 100X stock (see below)

PHOSPHATASE INHIBITORS
Sodium orthovanadate ($NaVO_4$): X1000 Stock solution,

1.83g in 10ml distilled water

Adjust to pH10 with concentrated HCl, the solution will turn orange.

Heat in microwave oven until almost boiling, solution will become colourless.

Cool to room temperature and adjust to pH10. Aliquot and store at -20°C.

PROTEASE INHIBITORS

Protease Inhibitor Cocktail Set I: Calbiochem (539131)

For 100X stock solution make up a fresh vial with 1ml sterile distilled water. Aliquot and store at -20°C. X1 solution contains 500mM AEBSF, 150mM Aprotinin, 1μM E-64, 1μM Leupeptin.

SODIUM FLUORIDE (NAF)

Make stock at 0.5M, final volume 10mls.

MW of NaF = 42 ∴ 1M = 42g/L

∴ 0.5M = 21g/L

Want final volume at 10mls ∴add 0.21g of NaF to 10mls of distilled water

POLYACRYLAMIDE GELS

Resolving gel: reagent volumes required for 30 ml, sufficient for 2 mini-gels
All volumes are given in μls

	Percentage gel	
Reagent	**8%**	**10%**
Distilled H_2O	13900	11900
30% Acrylamide Mix (Bio-Rad)	8000	10000
1.5M Tris pH8.8	7500	7500
10% SDS	300	300
10% Ammonium persulphate	300	300
TEMED	24	12

Stacking gel: reagent volumes for 5% gels for SDS-PADE (2 gels)
5mls final volume

Reagent	5%
H_2O	3100
30% Acrylamide Mix (Bio-Rad)	830
1M Tris pH6.8	630
10% SDS	50
10% Ammonium persulphate	50
TEMED (Bio-Rad)	3

1.5M TRIS PH8.8

90.84g Tris Base in 450ml distilled water
Adjust to pH8.8 with concentrated HCl
Make up to 500ml with distilled water and store at 4°C

1M TRIS-HCL PH6.8

6g Tris base in approx 6ml distilled water
Adjust to pH6.8 with concentrated HCl
Make up to 100ml with distilled water and store at 4°C

10% SDS

Dissolve 10g SDS in distilled water with gentle stirring and make up to 100ml.

10% AMMONIUM PERSULPHATE (APS)

100mg APS in 1ml distilled water.
Make fresh weekly.

RUNNING BUFFER
0.025M Tris Base
0.192 Glycine
0.017M SDS
Dissolved in distilled water, pH8.3 to 8.7
To make up dilute from X10 stock solution:
29g Tris Base
144g Glycine
10g SDS
Dissolve in 900ml distilled water, pH to 8.3 – 8.7 then make up to 1 litre.

TRANSFER BUFFER
0.012M Tris Base
0.096 Glycine
pH 8.3 to 8.7
20% Methanol added just prior to use
To make up dilute from X25 stock solution:
18.125g Tris Base
90g Glycine
Dissolve in 450ml distilled water mixing, pH to 8.3 to 8.7 then make up to 500ml.

LAEMMLI SAMPLE BUFFER (1:2)

Stock solution (Bio-Rad) with 5% 2-mercaptoethanol added just prior to use. Handled in fume cupboard at room temperature.

WASHING BUFFER (PBS/TWEEN)
PBS (Sigma) / 0.1% polyoxyethylene sorbitan monolaurate (tween-20) (Sigma P-1379)

BLOCKING BUFFERS
10% bovine serum albumen (BSA) in PBS / 0.1% Tween-20
5% Non-fat milk in PBS / 0.1% Tween-20
Made up for use, stored at 4°C

STRIPPING BUFFER
Restore Western Blot Stripping Buffer (Pierce). 10ml pre-made buffer incubated with membranes for 20 minutes at 37°C, with occational mixing. Membranes were washed well with PBS/0.1% tween-20 before re-probing.

APPENDIX III: RAW DATA FROM INHIBITION ASSAYS

Raw data from figure 3.2.1

A

	CD25-	CD62L+ Tregs	CD62L-Tregs
Healthy ctrl	$3.75X10^8$	$7.13X10^7$	$2.73X10^8$
Active RA	$2.79X10^8$	$1.03X10^8$	$1.65X10^8$
Infliximab	$3.61X10^8$	$2.09X10^8$	$6.85X10^8$

B

TNF- α			
	CD25-	CD62L+ Tregs	CD62L- Tregs
Healthy	3.35	0.885	2.05
Active RA	13.2	9.42	8.23
Infliximab	8.55	6.24	2.23
IFN- γ			
Healthy	3.165	0.81	1.86
Active RA	5.22	3.81	3.13
Infliximab	4.2	3.71	0.935

Mean raw proliferation (A) amnd cytokine (B) values raw values of percentage cytokine production of T effectors and T effectors co-cultured with either $CD62L^+$ or $CD62L^-$ Tregs.

Raw data for Figure 3.2.2.2

TNF-α								
CD25⁻	3.25	5.03						
CD62L⁺	0.87	3.82	0.81	1.28	1.96	1.60	0.78	2.46
CD62L⁻	2.96	1.42	2.37	3.92	1.30	2.66	2.47	4.17
IFN-γ								
CD25⁻	4.09	6.63						
CD62L⁺	1.73	3.82	1.02	1.66	1.31	3.18	0.98	3.15
CD62L⁻	3.77	1.79	2.74	5.11	2.16	3.12	2.58	5.90

Mean raw values of percentage cytokine production. Values shown in red are from infliximab-treated patients.

APPENDIX IV: LIST OF SUPPLIERS

Amersham Pharmacia Biotech
Amersham Place
Little Chalfont, Buckinghamshire, UK
Tel: 0870 606 1921

BD Bioscience
21 Between Towns Road
Cowely, Oxford
Tel: 01865 781688

Bio-Rad Laboratories Ltd
Bio Rad House
Hemel Hempstead
Hertfordshire, UK
Tel: 0800 181 134

Biowhittaker
1 Ashville Way
Wokingham
Berkshire, UK
Tel: 0118 979 5234

Dako Ltd
Denmark House
Angel Drove, Ely, Camebridgeshire, UK
Tel: 01353 669911

GE Healthcare
Amersham Place
Little Chalfont, Bucks, UK
Tel: 01494 542021

Insight Biotechnology Ltd (eBioscences)
PO Box
Wembly, UK
Tel: 020 8385 0303

Millipore UK Ltd
Units 3-5, The courtyards
Hatters Lane, Watford, UK
Tel: 087900 4645

Miltenyi Biotec
Almac House
Church Lane
Bisley, Surrey, UK
Tel: 01483 799 811

Pierce (Perbio)
High Street, Tattenhall
Cheshire, UK
Tel: 01829 771 744

R&D
19 Barton Lane
Abingdon Science Park, UK
Tel: 01235 529 449

Sigma Alrdrich
Fancy Raod
Poole, Dorset, UK
Tel: 0800 717181

Wallac
Crownhill Business Centre
Milton Keynes, UK

Zymed Labortories
561 Eccles Avenue
Soth San Francisco
California, USA
Tel: 00 01 800 955 628

POSTERS AND SEMINARS

Anti-TNF-alpha therapy in rheumatoid arthritis induces a population of regulatory T cells with a memory-like phenotype. (Poster presentation)
Nadkarni Suchita, Eddaoudi Ayad, Mauri Claudia, Ehrenstein Michael
Poster presented at the 16th European Congress of Immunology, Paris, France, September 2006.

Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. (Workshop seminar)
Nadkarni Suchita, Mauri C, Ehrenstein M
Oral presentation given at the World Immune Regulation Meeting, Davos, Switzerland, April 2007

PRIZES

Short-listed for the President's Evening Prize at the Royal Society of Medicine, June 2007

PUBLICATIONS

Nadkarni S, Mauri C and Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *Journal of Experimental Medicine, 2007 204 (1): 33-37*